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DIVISION OF CANCER BIOLOGY AND DIAGNOSIS

ANNUAL REPORT

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TABLE OF CONTENTS

	Page
EXTRAMURAL RESEARCH PROGRAM	
ASSOCIATE DIRECTOR	
Summary Report	1
<u>Tumor Biology Program</u>	
Summary Report	5
Grants	25
Contracts	53
<u>Immunology Program</u>	
Summary Report	55
Grants	71
Contracts	99
<u>Breast Cancer Program Coordinating Branch</u>	
Summary Report	103
Grants	115
Contracts	129
<u>Cancer Diagnosis Research Program</u>	
Summary Report	151
Grants	161
Contracts	167
Biology Support Contracts	189





## EXTRAMURAL RESEARCH PROGRAM

### DIVISION OF CANCER BIOLOGY AND DIAGNOSIS

The Extramural Research Program covers four scientific areas: Tumor Biology, Immunology, Diagnosis and an inter disciplinary Organ Site Program on Breast Cancer. These programs are primarily grant based, now that the conscious trend to convert research contracts to research grants has been accomplished. Only a few contracts remain, primarily concerned with support resources or screening efforts. During this fiscal year the program was streamlined further by the transfer of all radiological projects to the newly organized Radiology Program in DCT. In the closing months, Breast Cancer Program will join other organ site Task Forces in DRCCA, leaving this Division with three extramural program areas.

The budget this year reached \$138,000,000 of which 130,000,000 were in grant supported projects and 8,000,000 in contracts (including DCBD's share for the FCRF contract).

Within the overall purpose of defining key properties of cancer cells and tumors, three major areas of investigation are emphasized in Tumor Biology Program which correspond to different theories of how to control the development and progression of neoplastic disease. The first area is understanding the basic biochemical mechanisms involved in growth control (i.e., to understand how various biological signals initiate and maintain cell division). This information has lead to the development of specific hormonal and drug therapies. The second area is invasion. The invasive behavior of cancer cells is one of the first signs of malignancy. Malignant invasive cells can penetrate surrounding tissues, escape normal host immunological defense mechanisms in the bloodstream, and become established at multiple secondary metastatic sites of growth. Theoretically, if the invasive properties of malignant tumors can be controlled and these tumors confined to particular sites, then metastasis, the major killer in cancer patients, will not occur. The third area is developing detailed biological and biochemical information about processes which can induce cancer cell differentiation. If the genetic program of an actively growing cancer could be changed to terminal differentiation, then the malignant tumor would cease to divide and become harmless to the patient.

Improvements in technology are contributing dramatically and rapidly to the success of cancer research. The ability to routinely produce colonies of cells in culture (i.e., hybridomas) that can synthesize unlimited quantities of a single antibody molecule of desired specificity (i.e., monoclonal antibody) has revolutionized the use of antibodies as research tools. Furthermore, monoclonal antibodies are receiving increased attention as diagnostic tools and agents for improving immunotherapy. DNA recombinant technology has been perfected to the point where nearly any gene can be purified and studied under controlled experimental conditions. Our knowledge of gene regulation and genes responsible for growth transformation (i.e., "oncogenes") is increasing at a very rapid rate. This technology also has made possible the large scale purification of biological response modifiers which were available previously in only very limited quantities. The majority of basic research studies in cancer biology now relies on some aspect of hybridoma or DNA recombinant technology.

The Immunology Program supports three major areas of research: (1) basic immunology, i.e., how it works and what stimuli generate an immune response; (2) tumor immunology, i.e., the interaction between the tumor cell and the immune system which involves the effect of the tumor cell on the immune system as well as how the tumor cell responds to this interaction; and (3) mechanisms of immunologic intervention, i.e., how the immune system responds to intentional external perturbation for the benefit of the tumor-bearing host, for example, the use of bone marrow transplantation, monoclonal antibodies, interferon and thymic hormones.

The immune system is of interest to scientists conducting fundamental research in cancer because it plays a role in the body's defense against cancer. Many subsets of T and B cells are involved in the immune response to tumors. For example, the cytotoxic T cells, can have a direct killing effect on tumor cells, but many subsets of T cells have only regulatory functions, acting as helper or suppressor cells. Studies of basic immunology and regulatory networks have revealed much information on the regulation of the immune response to tumors. The availability of monoclonal antibodies has greatly enhanced the ability of scientists to study the individual parts and interactions of the immune response. Scientists are actively studying the role of the various types of immune cells both in the prevention of tumors (immune surveillance) and in the rejection of established tumors, primary and metastatic.

Recently developed hybridoma technology has had a significant impact on the identification of biologic markers and has been very important for the identification of tumor cell surface markers. For example, during the past year a number of laboratories exchanged and analyzed independently-produced antibodies to melanoma tumor cells. They tested these reagents for their functional reactivity and specificity to normal and tumor tissues; in addition, they attempted to define the antigens to which the monoclonal antibodies reacted. These antigens, although not entirely specific to melanoma tumor cells were found on most melanoma tumors and only rarely on non-melanoma tumor cells. Some reacted with a few normal tissues but to a lesser extent. Rigorous identification of antigens primarily associated with different types of human tumors is very important to human tumor immunology.

The Diagnosis Program emphasizes research in early detection, diagnosis, tumor localization, and monitoring changes during therapy or progression of disease. Projects in these areas are frequently concerned with improvement of existing techniques as well as the development of new tests and procedures. Many of the projects in the Cancer Diagnosis Research Program have begun in a more basic area such as, tumor biology or tumor immunology.

The major objective of the program is to recognize or detect cancer at the earliest possible stage to allow appropriate therapy to begin. Early detection and early treatment should improve the chances for the control of cancer, decrease mortality from the disease and increase survival and quality of life of those with cancer. Additionally, early detection is providing greater understanding of the natural history of different types of cancer in the early stages of disease. The Diagnosis Research Program consists of projects in five disciplinary categories: Biochemistry, Immunodiagnosis, Cytology, Pathology, and those projects that are clearly Multiple Disciplinary.

The objectives of the Breast Cancer Program are to promote and support basic multidisciplinary research projects in the laboratory and in the clinic, with the aim of increasing the understanding and improving methods regarding the etiology, epidemiology, diagnosis, prognosis, treatment or prevention of breast cancer. Through interaction with advisors, new and innovative research ideas are suggested, based on knowledge of ongoing research in breast cancer, and drawing upon the breadth of expertise represented, as well as upon information obtained at workshops and conferences in which the state-of-the-art on specific topics is presented. The staff of the Branch organizes the ideas into requests for investigator-initiated grant applications (RFA's) or program area announcements (PAA's), or requests for contract proposals (RFP's), depending upon the mechanism of funding considered most appropriate to achieve the goals of the project.

In addition, the staff publishes a monthly production of the publication "Intercom" which provides up-to-date listings of scientific papers on breast cancer, a list of meetings and conferences related to this disease and abstracts of presentations made at workshops.



## TUMOR BIOLOGY PROGRAM

### Description and Introduction

The Tumor Biology Program supports a broad spectrum of basic biological and biochemical research (See Appendix I) in the pursuit of one major goal, defining properties of cancer cells and tumors that uniquely distinguish them from normal, healthy cells and tissues. The supposition is that if we can define these properties and learn how to manipulate or change the biological signals responsible for the aberrant behavior of cancer cells, applied methods can be developed for the successful treatment, diagnosis and therapy of cancer patients. Within this goal are three major areas of investigation which correspond to different theories of how to control the development and progression of neoplastic disease. The first is understanding the basic biochemical mechanisms involved in growth control, whether these involve particular external signals that initiate the process of cell division or particular internal molecules more directly responsible for the control of DNA replication and metabolism. This kind of information can lead to the development of specific hormonal and drug therapies. The second is studying changes that occur at the molecular level which lead to cancer cell invasion. The invasive behavior of cancer cells is a prerequisite to malignancy, or the ability of tumors to invade surrounding tissues, escape normal host defense mechanisms and become established at multiple secondary metastatic sites of growth. Theoretically, if the invasive properties of malignant tumors can be controlled and these tumors confined to particular sites, metastasis, the major killer in cancer patients, will not occur. The third is to develop detailed biological and biochemical information about the processes which induce cancer cell differentiation. There is good reason to believe that many kinds of cancers will respond to external stimuli and differentiate. If the genetic program of an actively growing cancer could be changed to one of terminal differentiation, then the malignant tumor could be rendered harmless. Although the above emphases of the Tumor Biology Program in the areas of growth, invasion and differentiation is stated in simple terms, they provide a purposeful way of viewing the role of basic biological research to the ultimate goal of curing cancer.

The kinds of information developed in the Tumor Biology Program provide a foundation for and relate directly or indirectly to nearly every other program area within the National Cancer Institute. The importance of basic tumor biology research to the National Cancer Plan is reflected by the large \$56 million commitment of the NCI to this program area in FY 1982 (See Table I). Appendix II provides a complete listing of all grants supported by the Tumor Biology Program.

There are several trends which have characterized advancements in the Tumor Biology Program during the last six years. The first is that scientists have gone beyond the descriptive stages of cancer research. That is, they are no longer asking as many questions about what differences exist between normal and cancer cells, but instead are focusing on what these differences mean. For example, there is very little emphasis on what particular enzyme or isozyme in a tumor cell is produced abnormally, but there are major efforts underway to understand the molecular mechanisms that control enzyme synthesis and to locate the specific molecular sites within the cancer cell that are modified because of enzyme action. This example for enzymes applies to every other aspect of research investigating the aberrant behavior of cancer cells. The second is that genetics



and new genetic technologies are making rapid inroads into our understanding of how cells in normal tissues progress to become malignant cancers. Discoveries in molecular genetics, in particular, are telling scientists where to look for answers. Many disparate uninterpretable observations of the past are fitting into more focused, testable hypotheses. Clearly, the optimistic state of basic cancer research is due to the development and refinement of technologies such as DNA recombinant biology, monoclonal antibody production, and microinjection, as well as to the development of better model systems for studying cancer cell behavior.

We have emphasized a number of selected areas in which tumor biology research has demonstrated the greatest progress and/or promise in the last year. However, advancements in the genetics and molecular genetics of cancer are the most exciting and dominate the theme of this year's report.

### Selected Scientific Developments

#### A. Teratocarcinoma Workshop

On May 22, 23, and 24 the Tumor Biology Program conducted a workshop on Teratocarcinoma. This is a form of testicular or ovarian malignancy seen in both mice and men that apparently originates from the primordial germ cell. The tumor has been of interest to biologists for over 10 years who have developed experimental systems in which fundamental questions of cancer research could be addressed.

The intention of the workshop was to bring together about 40 pathologists, developmental biologists, virologists and cell and molecular biologists who work with teratocarcinoma-related systems to confront one another with their experimental data as well as their philosophical approaches. In addition, 20 scientists whose research experience dealt with other tumor cell systems were also invited to add another dimension to the discussions. The purpose was, further, to define the state of the art of teratocarcinoma research, including the advantages and limitations of the system, to stimulate interest in the molecular biology of the tumor cells, and, finally, to promote new collaborative efforts among the participants. The program provided for presentations of a series of "position papers" followed by break-up of the session's participants into four discussion groups, to allow for more intense exchange of information. At the end of the first and second days, the group reconvened for a plenary session in which each discussion group leader summarized highlights of the issues raised by his/her group.

The spontaneous teratocarcinomas that occur in patients as well as mice contain a heterogeneous population of cells, from the malignant stem cells to cell clusters with characteristics of heart muscle or bone or other differentiated somatic cells. Models of teratocarcinoma have also been developed (Stevens, CA 02662) by transplantation of normal mouse embryos of precise gestational age to the kidney capsule of recipient mice. A great number of cell culture lines have also been established



from the malignant stem cells in laboratories in the U.S. and England, utilizing both human and murine primary tumors as well as the extrauterine tumors. Dr. Stevens provided a list of transplantable tumors maintained at the Jackson Laboratory. The cell culture lines, called "Embryonal Carcinoma Cells" (ECC) are viewed as representative of the ultimate stem cell with the potential to differentiate into any cell type seen in an adult mouse. As such they have often been viewed as the prototype system for the study of differentiation, and for a test of the popular theory that cancer can be treated by converting malignant cells (which are always characterized histologically as abnormally immature cells) to normal, non-proliferating cells.

Davor Solter described data which extend the observations of Stevens on the development of experimental teratocarcinomas from 7-day old mouse embryos transplanted into extrauterine sites. Certain mouse strains such as BALB/c and C3H/He are "permissive" for forming malignant tumors while others, especially C57BL/6, are "non-permissive" and form mainly benign tumors. In addition, when the embryos are the result of matings between permissive and non-permissive strains and the recipients are similar F<sub>1</sub> hybrids, the outcome of the transplantation is markedly influenced by the maternal strain (Damjanov and Solter, 1982).

Perhaps the best known experiments involving teratocarcinoma cells were conducted by Beatrice Mintz and co-workers (Marx, 1982c). They demonstrated that single teratocarcinoma or EC cells could be injected into a normal mouse blastocyst, that the blastocyst could be reimplanted in an appropriate pseudo-pregnant mouse and that ultimately a normal mosaic mouse would be born that carried characteristic markers of the mouse strain from which the tumor cell was derived. Virginia Papaioannou presented the results of a literature survey she conducted to determine the success rate of experiments using this protocol, from several laboratories. Fifteen ECC lines were tested and the disappointing results showed that of 2137 blastocyst injections, 211 produced chimeric mice, 128 of which were normal and 83 of which were abnormal, an average 6% success rate. In only one female chimeric mouse thus far is there evidence that genetic information from the EC cell has also been integrated into the germ line and thus can produce subsequent generations of mosaic mice.

Dr. Papaioannou also described the several similarities between the embryo and EC cells, including the cell-cell interaction that leads to early parallel steps of differentiation and the inactivation of the X chromosome. In fact, the question of whether ECC provided any advantage over the embryo itself provoked active discussion. Dr. Papaioannou then pointed out the difference between teratocarcinoma as a model and as a tool. It is a model of early stages of normal embryonic development of the mouse and also of the association of differentiation with loss of proliferative capacity. As a tool it is a source of mutants with different cell surface antigens and different developmental capacities and is capable of forming chimeric mice which might be used in disease-model production (Papaioannou, 1981).

Elwood Linney (Fujimura and Linney, 1982) described experiments in molecular biology he has ongoing in his laboratory to integrate new genes into the chromosomes of ECC which in turn might be introduced into blastocysts, ideally

to select for offspring with germ line contributions of the tumor cell that carried along the artificial gene. Also of emphasis is the comparison of EC cells with their differentiated derivatives in their ability to express newly introduced genes.

In the laboratories of Eileen Adamson (Hogan et al., 1981) and of Sidney Strickland, emphasis is on physical and chemical means of inducing the differentiation of one ECC line, the F9, that does not spontaneously differentiate. Dr. Strickland has demonstrated that retinoic acid can promote monolayers of the F9 cells to form primary endodermal cells that represent a branch point from which cells with different phenotypes can emerge. The second step of the induction is critically dependent on environmental conditions; cells allowed to remain in contact with one another aggregate and organize into a structure resembling visceral endoderm, while cells treated with cAMP form parietal endoderm. The distinction between these types of endoderm includes morphology and more subtle biochemical changes that require sophisticated probes for either alpha-fetoprotein or basement membrane proteins (laminin, type IV collagen and fibronectin), respectively. However more precise markers such as cell surface antigens are needed to better define the transitions from one differentiated state to the next. New technology that allows for biochemical analysis of single cells will also be of great value in studying the mechanism of differentiation (Strickland, 1981).

The final workshop session emphasized the use of human teratocarcinoma and the ECC derived from them. Currently no multipotent cell lines comparable to the murine lines are available. The only discernible change inducible in human ECC is morphological, from a rounded to a flat cell. In contrast to the mouse lines, most of which have a near normal karyotype, the human tumor cells are not diploid. Chris Graham (Oxford University), who led this discussion, also provided a comprehensive list of human xenograft tumor lines that have been described in the literature.

Barry Pierce (CA 15823) whose overview of the workshop closed the meeting was highly optimistic about the future of teratocarcinoma research. He pointed out that this system has demonstrated that a highly malignant cell population can be re-programmed into an organized series of differentiating cells. He believes that if an embryonic field can regulate a cancer then we can exploit that system to help us develop new modes of treatment for victims of cancer.

## B. Tumor Cell Differentiation

Teratocarcinoma is not the only model of cancer that has encouraged the idea of treating malignancies, not by killing the cells, but by changing them into mature cells. In leukemia, for example, the patient's blood cell count is increased about 10 times and the majority of cells present are abnormally immature. Cells from the HL60 cell line, derived from a patient with promyelocytic leukemia, undergo differentiation to granulocytes when treated with DMSO, to cytotoxic macrophages when treated with phorbol esters (Rovera et al., 1982) and to myelocytes when treated with beta interferon. Cells from the line K562, developed from blood samples from a chronic myelogenous leukemia patient, acquire characteristics of reticulocytes and cease proliferation when exposed to the interferon inducer, double-stranded-RNA.

Some limited success has also been seen in leukemia patients in whom the double-stranded-RNA promotes maturation of the blood cells (Strayer et al., 1981). The powerful influence of small molecules, especially derivatives of vitamin A (retinoic acid), on the differentiation of epithelial cells has been widely described. In the case of cultured keratinocytes newly derived from human epidermis, the removal of vitamin A from the culture medium leads to cellular alterations characteristic of terminally differentiating epidermis. These include loss of cell motility, increased cell adhesiveness and synthesis of very large keratins typical of stratum corneum. It is clear that the effects of vitamin A govern an extensive program of differentiation, which may relate to altered keratin synthesis in neoplastic keratinocytes (Fuchs and Green, 1981).

#### C. Tumor Angiogenesis Inhibitor

Most solid malignant tumors have the capacity to induce the generation of new capillary blood vessels (i.e. angiogenesis) and become surrounded with a relatively independent blood supply, which is necessary for continued growth. Without this blood supply, many tumors will not only cease to grow but in some cases regress. A capillary system surrounding a tumor has two major implications in the control of tumor growth and progression toward the malignant state. The first is that without a sufficient supply of nutrients, tumor growth must be slower. Secondly, the close proximity of blood vessels to the tumor provides ready access of invasive tumor cells to the bloodstream and thus increases the probability of metastasis. The major approach to learning how to control tumor angiogenesis has been to purify stimulatory factors made by tumors and inhibitory factors from tissues such as cartilage, which are resistant to angiogenesis. Unfortunately, these protein factors are present in very small quantities and have proved to be extremely difficult to purify. Work supported in the last seven years by the National Cancer Institute has not resulted in the isolation and purification of any of these proteins.

Despite the lack of success in purifying angiogenesis factors, the information developed has led to a new approach to the problem. A major observation was that angiogenesis inhibitor proteins possessed an unusually large number of positive charges. It was reasoned that inhibition might be accomplished simply through a charge effect. The qualities of a good inhibitor would be its ready availability, high positive charge, and relatively few toxic side effects when administered. Protamine, a small arginine-rich (and thus positively charged) protein found only in sperm, appears to satisfy most of these requirements (Taylor and Folkman, 1982). Investigators supported by the Tumor Biology Program discovered that protamine effectively inhibits capillary proliferation observed in the growth of embryos, in inflammation and certain immune reactions, and in the growth of solid tumors implanted in the rabbit cornea. While protamine satisfies the criteria of availability and positive charge, it unfortunately produces lethargy, weakness and occasional sudden death in laboratory animals if given in high doses. Protamine probably will not be useful for therapy, but it should be a valuable tool for studying the mechanism of angiogenesis and could point the way toward the development of better



therapeutic agents. For the first time there is the real possibility of designing angiogenesis inhibitors that are more potent and less toxic and which can be used in conjunction with conventional chemotherapeutic agents. Prevention of angiogenesis could have an extraordinary effect on cancer treatment.

#### D. Tumor Cell Invasion and Metastasis

A major emphasis of this program has been to identify the properties of cancer cells responsible for invasive, migratory behavior. Without the development of invasive properties, cancers would not metastasize and would become far less lethal to the patient.

Most cells are surrounded by an insoluble protein coat called the extracellular matrix to which cells adhere. Cancer cells often lack a well-defined extracellular matrix and they also fail to form specialized matrix structures referred to as basement membranes. The lack of adhesive surface matrices and surrounding basement membranes is believed to be important for the ability of cancer cells to invade surrounding tissue and metastasize to distant sites. Fibronectin is one of the major components of the matrix and has been studied extensively. This glycoprotein appears to be important for the assembly of extracellular matrices and may be a key component of the cellular adhesion process. There are specific sites on the molecule (i.e. domains) which attach to cells and various components of the matrix such as glycosaminoglycans, fibrin and collagen (Pierschbacher et al., 1981; Ruoslahti et al., 1981). Fibronectin is a prototype molecule for proteins involved in cellular adhesion and understanding the structure and function of this protein may help us develop ways of controlling the spread of cancer.

Cancer cells might be regarded as antisocial in that they grow in the wrong places at the wrong time and don't listen to the signals that control the behavior of normal cells. The aberrant interaction of cancer cells with fibronectin may contribute significantly to the properties responsible for the malignant phenotype of tumors. Several years ago it was reported that fibronectin matrices isolated from normal cells could restore tumor cells in culture to a more normal morphology and social behavior. This observation has been carried further to see how different kinds of tumors respond to fibronectin matrices. In general, single cell suspensions from sarcomas (tumors of connective tissues) respond to these matrices by forming large tissue-like aggregates. Thus, fibronectin appears to restore adhesive properties to these cells. However, cells from carcinomas (tumors of epithelial tissue) rarely aggregate under these conditions. Only a few kinds of sarcomas could destroy fibronectin, but nearly all carcinomas were capable of enzymatically digesting fibronectin. Interestingly, only those sarcomas which were metastatic acted like carcinomas in their interaction with fibronectin. Since 85% of all cancers are metastatic carcinomas, there appears to be a very strong correlation between metastasis and the inability of cancer cells to aggregate in the presence of fibronectin and the ability of cancer cells to degrade fibronectin (Hsieh and Chen, 1982). These results are supportive of the idea that one way metastatic cancer cells disregard signals responsible for cellular adhesion and begin migrating to distant locations is by producing enzymes that destroy the physical integrity of extracellular matrices.

Efforts to detect qualitative differences in the surface protein components of cells with different metastatic potential have been disappointing. There are, however, variants of the B16 melanoma mouse model which have been selected for high and low metastatic potential. Using these variants, it clearly has been shown that the molecules which impart high metastasis are present in the cell surface. Recently, it has been demonstrated that the metastatic potential of B16 melanoma variants correlates with specific cell surface alterations (Rieber and Rieber, 1981). An explanation consistent with these results is that cells with lower metastatic potential have greater protease and/or glycosidase activity, which in turn leads to greater turnover of surface components required for cell-cell interaction and subsequent cell survival during the invasive process. This interpretation of results suggests a theory opposite to that proposed from observing metastatic cell interactions with fibronectin and emphasizes some of the difficulties scientists have had in developing a unified theory for explaining the invasive and metastatic processes.

A recent observation which potentially has some significance is that mutations of a transforming virus can substantially enhance the metastatic potential of resulting transformed cells (Dixon et al., 1982). Is this another example where viruses will help us in the study of cancer cell behavior? If so, for the first time, it may be possible to correlate specific genetic differences (in viruses) to differences in cellular parameters that determine metastasis.

#### E. Growth Factors

Normal cells growing in culture always require classical hormones, protein growth factors or both. Comparable transformed or malignant cells in culture somehow escape from normal growth regulation and frequently lose their dependence on these growth factors. Research efforts into the mechanism of action of two growth factors, platelet-derived-growth-factor (PDGF) and epidermal growth factor (EGF), have provided exciting new information that may relate directly to the biology of tumor growth.

PDGF is a potent mitogen for fibroblasts and smooth muscle cells and has been implicated in normal wound healing. It has been demonstrated that this growth factor induces the synthesis of at least five specific cellular proteins believed to be involved in the signal for growth. A tumorigenic, transformed fibroblast that has lost its PDGF requirement appears to synthesize several of these proteins constitutively, independent of the factor (Pledger et al., 1981).

Results of studies from other laboratories are consistent with the hypothesis that loss of growth control is the result of constitutive expression of critical gene products. Cell fusion experiments have indicated that a single cell primed with PDGF can trigger DNA replication in a non-treated fusion partner, suggesting that the target cell had formed some "second signal" in response to the priming. The "second signal" is apparently the product of one or more specific genes whose expression is induced by the PDGF in normal cells. These DNA sequences have now been isolated and cloned and can be used as probes for malignant cells to determine if their

activity is expressed and if the expression is continuous. Such experiments are of considerable significance since the "oncogenes" discussed in Section I may also be growth regulatory genes transposed in chromosomes to sites where they are constantly expressed (Smith and Stiles, 1981).

Another mechanism that could explain why tumor cell growth is independent of PDGF is that the cells produce their own growth promoting factors. Studies with a line of human osteosarcoma cells have demonstrated the presence of PDGF-like polypeptides in the culture medium in which the cells are grown. These polypeptides are active in promoting growth of normal fibroblasts and they cross-react with antisera prepared against true PDGF (Antoniades and Owen, 1982). Characterization of these osteosarcoma growth factors may be especially important in relation to other autocrine factors that have been described by George Todaro.

Certain parallels can be drawn between the results of studies with PDGF and with epidermal growth factor. Although the target cell is distinctly different (EGF is an epithelial cell mitogen), transformed cells also escape from growth control by EGF. And there is evidence that transformed cells in culture may produce their own EGF-like growth factor. Since EGF has been sequenced and is commercially available, comparative studies with the tumor cell factor may soon be forthcoming. Other research efforts have focused on the cell surface receptor for EGF and the mechanism whereby the receptor autophosphorylates itself in the presence of EGF. Highly specific antisera prepared against different forms of the receptor precipitate both EGF-binding activity and the associated EGF-sensitive kinase activity, and the data support the idea that both activities are localized in one membrane glycoprotein. The kinase is of special interest since it has an unusual specificity for phosphorylating only tyrosine moieties, similar to that of the transforming kinases of tumor viruses. Attempts are underway to demonstrate a casual relationship between the phosphorylation of intracellular substrates by EGF and growth control of intact cells (Carpenter et al., 1982).

#### F. Regional Differences and Tumor Growth

A review of scattered information in the scientific literature over the past 46 years has revealed some rather striking but consistent regional influences on the patterns of growth of normal and neoplastic cells in animals (Auerbach and Auerbach, 1982). In both the mouse and the rat, except for a few strains, carcinogens induce tumors more frequently anteriorly than posteriorly (i.e. toward the rear of the animal). Also, it has been found that, regardless of the tumor type, (i.e. sarcoma, melanoma, teratocarcinoma, etc.) transplanted tumors, whether inoculated intradermally, subcutaneously or intraperitoneally, demonstrate strikingly greater growth and development anteriorly. An interesting observation, however, is that when tumor cells are adapted to growth in liquid as single cells in suspension rather than as solid tissues anteriorposterior polarity for growth is lost. Thus, it appears that without cell adhesion there is no manifestation of regulatory factors associated with the local tissue environment, and regional influences on growth disappear. In rodents, the incidence of spontaneous mammary tumors and their rate of development was greatest in



the anterior mammary organs, as long as tumor viruses were not present. Thus, somehow tumor viruses can disrupt regional controls of growth. Although it could be postulated that some unique properties of tumors account for preferential growth and development, this apparently is not the case. Normal tissues when transplanted to anterior sites grow better. In addition, there are several aspects of wound healing that demonstrate clear topographical preferences. Both cell division and cell migratory activity are greater anteriorly than posteriorly. Interestingly, these are both properties of metastatic cancer cells. There appears to be a strong correlation of all of the above with differences in microcirculation in the anterior and posterior regions of the animal. Differences in vascular system development are very likely related anteroposterior differences in the development of the nervous system which exerts considerable control over blood vessels. The role of blood vessels as regulators of tumor growth is not a concept that has gone unnoticed. Tumors, when they produce tumor angiogenesis factors, which induce a blood vessel supply to the tumor, may be directly upsetting normal developmental biochemical gradients in order to achieve a selective growth advantage. The above observations are important for two reasons: first, understanding the basic biological mechanisms which underlie regional differences in cell growth patterns may provide valuable insights into the control of tumor growth; second, and perhaps of more immediate practical significance, is that without careful consideration of these regional differences in the design of experiments examining tumor growth and metastasis in animals, scientific results may be gravely misinterpreted.

#### G. Cancer Genetics - Cell Chromosomes

Though the hypothesis that cancer must be caused by some abnormality in genetic material has always seemed tenable, on examination, the chromosomes of half the patients with cancer appeared to be entirely normal. Now cytogeneticists are examining cancer cells with a powerful new technique and discovering that almost 100% have detectable abnormal chromosomes. This important information includes studies on patients with chronic myelogenous leukemia, acute non-lymphocytic leukemia, acute lymphocytic leukemia, lymphoma, and carcinoma of the breast, nervous system, lung, ovary, cervix and colon. Of equal importance is the demonstration that a specific defect can be identified for most types of malignancy that is consistently seen in 2/3 of patients. In most solid tumors and some leukemias and lymphomas there is a deletion of a chromosomal segment, indicating an actual loss of genetic material. But in the majority of leukemias and lymphomas there is, rather, an exchange of segments between chromosomes at precise breakpoints such that genetic information must be rearranged (Yunis et al., 1981). Specifically, it has been reported that in all patients with acute promyelocytic leukemia examined, a segment of chromosome #15 is translocated to #17. This translocation is not seen in other types of cancer. Additionally a #9 to #22 chromosomal translocation is identified with chronic myeloid leukemia, and a #21 to #8 exchange with acute myeloblastic leukemia. In Burkitt's lymphoma and B-cell acute lymphocytic leukemia the translocations involve chromosomes #8 to #14 and the joining point on #14 is a new focus of investigation. Gene mapping data indicates that immunoglobulin genes are located on that chromosome in the area of the attachment point of the segment of #8

(Rowley, 1982). The relocation of any genes to chromosome sites with better access to an "on" switch may provide a selective advantage to a cell in terms of growth. Such a mechanism may be invoked to explain the so-called "oncogenes".

In studies of children with acute lymphocytic leukemia, four categories of karyotype were observed: normal, hyperdiploid, hypodiploid and chromosomal rearrangements, and there is some indication that the prevailing karyotype in a leukemia patient may help predict the child's prognosis (Heerema et al., 1982). Other data indicates that within certain solid tumors, more than one cell population can be identified, each with its own specific chromosomal pattern. It appears that, under culture conditions, certain of these subclones is more drug resistant than others, suggesting a possible approach to improving cancer therapy (Trent and Buick, 1981).

#### H. Cancer Genetics - Gene Amplification

A major focus of molecular genetics in the last twenty years has been on the modulation of gene expression. During development and differentiation, cells in complex organisms in order to diverge functionally must selectively express only a limited percentage of the total genes available. Mechanisms that control selective gene expression allow different kinds of cells (e.g. nerve cells, muscle cells) to modulate the dosage of various gene products. One way to modulate dosage is to regulate the rate of expression of a given gene and this area of research has received the greatest attention. However, regulation of gene expression may also occur by amplifying or reducing the number of genes available for expression. Research in the area of gene amplification has received attention in the last few years because scientists have discovered that cultured animal cells and human tumors can selectively amplify specific genes to become resistant to drugs. Furthermore, there are genetic abnormalities in cancer cells, which have been shown in some cases to represent regions of gene amplification, that appear in two forms, as extrachromosomal elements called double minutes (DM) and as expanded regions of existing chromosomes called homogenous staining regions (HSR). Two different areas of investigation, chromosomal abnormalities and drug resistance from gene amplification, appear to be converging and may be of greater significance to cancer cell behavior than previously believed (Schimke, 1982).

The phenomenon that allows cancer cells to become resistant to drugs is of extreme importance because acquisition of resistance to chemotherapies is a major problem in cancer treatment. Scientists are now convinced that drug resistance develops as a result of gene amplification. One of the most common drugs used in the treatment of cancer is methotrexate or MTX. Another newer drug is N-phosphonacetyl-L-aspartate which is abbreviated PALA. Both MTX and PALA specifically inhibit the enzymes dehydrofolate

reductase (DHFR) and aspartate transcarbamylase (ATCase), respectively. These enzymes are critical for the cellular synthesis of components that make up DNA and, when completely inhibited, cells such as cancer cells are prevented from dividing. However, cancer cells which have become resistant to MTX (Melera, 1982) and PALA (Padgett et al., 1982; Wahl et al., 1982) exhibit additional copies of the DHFR and ATCase genes. Somehow the cell has learned to increase the dosages of these enzymes by amplifying the gene responsible for their synthesis. As more enzyme molecules are produced, the effective inhibiting concentration of MTX and PALA becomes considerably higher. Clearly, learning how cells amplify genes to gain a selective growth advantage in the presence of toxic drugs is a critical area of tumor biology research. If the mechanism of gene amplification is commonly used by cancer cells, it may have major implications in many other areas of cell behavior.

There are reasons to believe from results reported by other scientists that gene amplification occurs in situations other than those involved in drug resistance. Two mouse adrenal tumor cell lines have been developed from the same tumor, one which carries DM's and another which contains HSR's. Both the DM's and HSR's from these two different cell lines result from amplification of the same gene (George and Powers, 1981; George and Powers, 1982a). A virally transformed mouse 3T3 cell line also contains DM's but different genes are amplified (George and Powers, 1982b). These results are of extreme importance because they indicate specifically that different genes may be amplified in different tumor cells and suggest generally that gene amplification can occur for many different genes.

## I. Cancer Genetics - Oncogenes

The most exciting and potentially most important area of cancer biology research has been the discovery that there are specific genes in viruses, DNA from normal cell lines, and DNA from cancer cell lines which can transform 3T3 cells in culture (Rigby, 1982; Marx, 1982a; Marx 1982b). Transforming genes have been referred to as "oncogenes" and experiments with oncogenes suggest a much closer relationship than previously believed between cancers of viral and nonviral origin. These results have been possible because of two major advancements in technology: (1) the refinement of DNA-mediated gene transfer techniques and (2) the use of DNA recombinant techniques of genetic engineering.

In the last year a number of laboratories independently examined several cell lines derived from different kinds of cancers to assess the presence or absence of transforming genes. Basically, these experiments were conducted by taking pieces of DNA from the cancer cells, transfecting mouse NIH 3T3 cells in culture and then looking for altered growth patterns (i.e., transformed behavior) of the 3T3 cells. Using this assay, oncogenes have been detected in chicken B-cell lymphomas, human and mouse bladder, human lung and mammary carcinomas and human and mouse B and T-lymphocyte neoplasms (Krontiris and Cooper, 1981; Lane et al., 1982). These neoplasms included spontaneous-occurring, chemically induced and virally induced tumors. The oncogenes from mammary carcinomas, regardless of whether



they originated from human or mouse tumors, appeared to be very similar, but clearly different from those derived from lymphoid neoplasms (Lane et al., 1981). Oncogenes from human bladder and lung carcinomas were homologous to the ras genes of Harvey and Kirsten sarcoma viruses (Dir et al., 1982). Three different oncogenes were found in human colon carcinoma, bladder carcinoma and promyelocytic leukemia cell lines (Murray et al., 1981). Transforming genes identified from human lung and colon carcinomas were closely related but different from those in bladder carcinoma and neuroblastoma (Perucho et al., 1981). Also, it has been demonstrated that non-transformed cell lines (i.e., "normal cells") contained latent transforming genes which could transform 3T3 cells at a high frequency under the proper conditions. The conclusions for the results have been that (a) oncogenes are usually tissue specific but not always; (b) homologous counterparts to oncogenes exist in normal cells; (c) oncogenes of a given tissue type may not be species specific; (d) there are a limited number of different oncogenes; and (e) some oncogenes are related to viral transforming genes.

The next set of observations using the same kind of assay for transforming activity in the NIH 3T3 system was that latent transforming genes present in some normal cells (i.e., proto-oncogenes) could be activated if attached to the control element of a viral oncogene. In addition, normal cell lines were clearly shown to possess DNA segments homologous to viral oncogenes (Chang et al., 1982). Thus, the relationship of oncogenes from both normal and cancer cells to some viral transforming genes and to each other has been confirmed in two ways: (1) by studying cancer cell DNA through transfection experiments (2) by using viral oncogenes and control elements in transformation experiments.

The factors which actually convert proto-oncogenes of normal cells to activated oncogenes of cancer cells or viruses remains unknown. However, in most cases examined so far, there is no indication that the transformed state arises by any major rearrangements of the genetic material. Experiments in the future are likely to pursue the possibility of a mutational or minor rearrangement event that alters the transcriptional control of proto-oncogenes. However, there is also preliminary evidence with bursal lymphomas of the chicken that suggest the presence of oncogenes in highly repeated sequences as if they were amplified. Regardless of how oncogenes become active, the most important conclusion to develop at this time is that cancer is an aberration of gene regulation leading to the synthesis of normal gene products in excessive amounts at the wrong time. This may also explain why years of past research have failed to turn up a single tumor-specific gene product.

Although these results are exciting and have revolutionized many of our current concepts of the cancer process, there are a number of areas that need to be resolved. First, what is the general validity of the NIH 3T3 cell assay for cellular oncogenes? In the final analysis we need to know whether an oncogene will transform its parent cell type. Second, how can we resolve our previous concepts of a multi-step carcinogenic process with this single event transforming assay? Third, when will

proto-oncogenes and oncogenes be described in normal tissues and primary tumors, respectively? So far "normal cells" have been cell lines propagated in culture and by their very nature could be regarded as abnormal. Efforts must be made to show that results which have been reported to date are not an artifact of cell culture.

Considering the above reservations, we appear to be on the verge of being able to isolate, clone, and characterize the transforming genes and gene products of cancer cells.

#### J. Cancer Genetics - Cell Hybrids

A debate has been ongoing for years as to whether malignancy is a dominant or recessive trait, that is, can a transforming modification in a single chromosome render a cell malignant or will that chromosome's normal, like pair dominate and suppress tumor behavior? A powerful tool that is being utilized to approach this question is somatic cell hybridization. Two parent cells (either normal or malignant) are fused together to make a hybrid cell who's behavior should imitate one or the other of the parents. Results of many of these studies have indicated that tumorigenicity is suppressed when a normal cell is fused with a malignant one, however, other investigators have found that tumorigenicity is expressed dominantly in such hybrids. The major reason for the differences found in these studies is that both intraspecific (rodent + rodent) and interspecific (human + rodent) hybrid cells are often chromosomally unstable, i.e. as the cells replicate they lose ("segregate") chromosomes, and their behavior keeps changing. In attempts to improve hybrid analysis several laboratories are now focusing on intraspecies hybrids, especially human, in which the chromosomal number and the phenotype are fairly stable.

Among the problems confronting all biologists who must score cells as normal or malignant is that of a reliable assay. In vitro phenotypic changes in a cell, including loss of density-dependent inhibition of growth, reduced requirement for serum growth factors, altered metabolic rates, expression of new products and cell surface antigens and anchorage independence, are all associated with malignancy. It has been suggested that transformation is a progressive process requiring more than one step from normal to malignant, probably controlled by multigenic events (Stanbridge et al., 1982; Sager, 1982). The appearance of these phenotypic traits in vitro may represent the first step, which is necessary but not sufficient for tumorigenicity. The ultimate test of malignancy is the ability to form an active tumor in a intact animal.

Using this assay, results of hybrids between normal human fibroblasts + tumorigenic human HeLa cells have been unequivocal -- 100% failed to form tumors in recipient animals. However even these hybrids did not have 107 chromosomes as would be predicted (46 from the fibroblast + 61 from the HeLa cell line), and after extended periods of time in culture, isolated clones of tumorigenic cells did appear. This reexpression of tumorigenicity seemed to be associated with the loss of chromosomes

number 11 and 14. Interestingly, when the karyotype of the malignant parent cell is also close to diploid (46 chromosomes) the hybrids formed cannot even be maintained in culture but act like normal cells with a limited lifespan (Stanbridge et al., 1981).

Further extensions of these experiments have been carried out to determine whether one tumor cell can complement a tumor cell of a different type. Crosses between two carcinoma cell lines resulted in hybrid cells that were highly malignant. In contrast, fusions between carcinoma cells and sarcoma cells, or between carcinoma cells and melanoma cells resulted in hybrids that were nontumorigenic, i.e. the malignant characteristics were suppressed. These results suggest that different loci govern malignancy in different tumor types, perhaps a distinct one for each somatic cell type.

Although the critical role of the nucleus has been established in a variety of experimental systems, the role of the cytoplasm may also be important in the malignant behavior of cells. An important experimental approach to this issue is a hybrid system in which one of the two fusion parents lacks a nucleus and various combinations of whole cells plus "cytoplasts" from rats and mice, both transformed and normal, are tested. Unfortunately induction as well as suppression of phenotypic traits associated with malignancy were observed and no correlation could be established between in vitro behavior and tumor formation in mice. (Sudilovsky, CA 25164)

Another type of cell hybrid can be produced by transplanting a nucleus from one cell into an enucleated cytoplasm prepared from another cell. In experiments with normal parent cells representative of connective tissue, liver, erythrocytes or fibroblasts, both nucleus and cytoplasm were able to exert specific regulation on the hybrid causing both expression and repression of characteristics. (Hightower and Lucas, 1982)

## Discussion

Advancements in basic biological research have made major contributions to our understanding of the cancer process in the last five years. Recent discoveries have led to new, more focused experimental hypotheses concerning the aberrant behavior of cancer cells. Five years ago we did not know that the extracellular matrix was composed of specific molecules that regulate cell adhesion and migration. We now realize the potential importance of the extracellular matrix for understanding and controlling the invasive behavior of cancer cells. Through technical advancements in genetics, we are now able to combine knowledge developed in bacterial systems and eukaryotic virus systems with new advances in mammalian genetics to ask specific questions about the regulation of gene expression in cancer cells. The discovery that there are a limited number of oncogenes which are mostly tissue specific and can be related to viral transforming genes is a major breakthrough. The identification of oncogene products has already progressed in one system to the point where we know that a viral oncogene produces a very special kinase that phosphorylates tyrosine residues, has homologous forms in normal human fibroblasts (Shealy and Erikson, 1981), and phosphorylates an important protein believed to link the cell surface to



cell internal organizational structures (Hynes, 1982) known to be disrupted during transformation. Thus, we know that viral oncogenes have counterparts in normal human cells which, when activated, can upset the normal balance of protein phosphorylation in the cell. There are also intriguing similarities between how some growth factors and oncogene kinases stimulate tyrosine specific protein phosphorylation. What once were disparate observations between studies of disorganization of cell architecture during transformation, stimulation of cell growth by growth factors, and transforming genes in viruses can now be combined into more unified concepts concerning cell growth control. Clearly, protein phosphorylation has become recognized as a major general mechanism that regulates intracellular events in mammalian cells and there is reason to believe that many different cellular functions are controlled by common protein kinases (Cohen, 1982). Further significant progress in understanding how different oncogenes transform cells and how transformed cells invade the extracellular matrix should be made in the next few years.

There are sure to be many surprises in the future. However, attempts to set priorities in the past on what aspects of basic research are important or unimportant to tumor biology research has taught us an important lesson. For example, three years ago, viruses were believed to be less important to cancer, yet recent studies of how viruses transform cells have been major contributors to understanding oncogenes in human cancers and establishing that cancer is caused by increasing the dose of a normal cell product rather than stimulating the formation of a cancer specific product. Revitalization of studies of gene amplification processes, which contribute to cancer cell drug resistance, may also be important to transformation and tumor progression. Any genetic mechanism which allows a cancer cell to adapt to its environment in order to gain a selective growth advantage is important to study. It is quite possible that increasing the dose of normal gene products occurs by increasing the rate of expression of a given gene, as already demonstrated, by gene amplification or by genetic mechanisms not yet discovered. Our whole concept of the stability of the mammalian genome has changed remarkably in the past few years. The discovery of transposable genetic elements has explained how diversity is generated through gene rearrangement to produce, under controlled conditions, different antibody molecules of varied specificity, or, under different conditions, the cancerous phenotype. Last year, we concluded that tumors in plants were of little relevance to mammalian cancer processes because they were caused by a bacterial plasmid. But it has recently been discovered that plasmids are generated from mammalian DNA (Calabretta et al., 1982) and that plasmids may be the way in which genes travel back and forth between species barriers (Lewin, 1982).

As quoted from two recent reviews: "The argument that fundamental research in molecular biology is irrelevant to the cancer problem is truly dead and buried" and "But at least cancer researchers now know where to direct their efforts, and no one can criticize the investment of money for cancer research in basic molecular biology."

TABLE 1

FISCAL YEAR 1982

## TUMOR BIOLOGY PROGRAM

## SUMMARY BY SUB-CATEGORY (DOLLARS IN THOUSANDS)

	NON-COMPETING		COMPETING		TOTAL	
	No.	Amount	No.	Amount	No.	Amount
A Cell Surface	60	\$6,043	37	\$3,379	97	\$9,422
B Enzymes	28	2,408	4	492	32	2,900
C Peptide Hormones	10	1,026	6	605	16	1,631
D Steroids	19	1,935	8	674	27	2,609
E Membranous Organelles	8	1,340	4	543	12	1,883
F Ribosomes & Polyribosomes	3	634	1	207	4	841
G M-RNA	15	1,804	3	297	18	2,101
H T-RNA	7	628	2	115	9	743
I DNA	10	1,069	2	178	12	1,247
J Growth Factors	15	1,898	8	739	23	2,637
K Nucleus	14	1,326	6	528	20	1,854
L Contractile Elements	7	765	4	366	11	1,131
M Development & Differentiation	49	5,236	15	1,484	64	6,720
N Cell Growth, Cell Division	24	2,294	7	564	31	2,858
P Somatic Cell Genetics	10	1,334	9	979	19	2,313
Q Inheritance of Neoplasms	3	154	3	318	6	472
R Plasmids, Viruses	6	593	3	217	9	810
S In Vivo & In Vitro Tumor Lines	8	890	3	322	11	1,212
W Difficult to Classify	5	720	3	293	8	1,013
Sub Total	301	32,097	128	12,300	429	44,397
V Program Projects	13	8,006	5	3,317	18	11,323
T Conferences	1	89	4	139	5	228
Sub Total	14	8,095	9	3,456	23	11,551
Total	315	40,192	137	15,756	452	55,948

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- R01-CA-12150    Unusual Lipids in Cancer Tissues  
Baumann                      University of Minnesota at Austin
- R01-CA-12306    Role of Cell Surfaces in Initiation of Cell Division  
Cunningham                      University of California, Irvine
- R01-CA-12753    Cell Surface Glycoproteins  
Grimes                      University of Arizona
- R01-CA-12790    Membrane Transport Adaptations by Human Lymphocytes  
Lichtman                      University of Rochester
- R01-CA-12920    Mechanisms of Intercellular Adhesion  
Oppenheimer                      California State University, Northridge
- R01-CA-13402    Surface Membranes in Normal and Cancer Cells  
Atkinson                      Yeshiva University
- R01-CA-13605    Chemistry and Measurement of Intercellular Adhesion  
Steinberg                      Princeton University
- R01-CA-14431    Cell Adhesion of Normal and Malignant Liver Cells  
McGuire                      National Jewish Hospital and Research Center
- R01-CA-14464    A Program on the Etiology and Diagnosis of Cancer  
Lowenstein                      University of Miami
- R01-CA-14496    Metal Ion Activation of Lectins  
Magnuson                      Washington State University
- R01-CA-14551    Penetration of Macromolecules into Mammalian Cells  
Ryser                      Boston University
- R01-CA-14764    Glycolipid Metabolism in Tumor and Transformed Cells  
Basu                      University of Notre Dame
- R01-CA-15047    O-Alkyl Lipids in Surface Membranes of Tumor Cells  
Friedberg                      University of Texas Hlth. Sci. Ctr., San Antonio
- R01-CA-15483    Biochemistry of Mucopolysaccharides  
Davidson                      Pennsylvania State University Hershey Medical Center
- R01-CA-16335    Cell Junction Formation and Role in Cell Proliferation  
Sheridan                      University of Minnesota of Minneapolis-St. Paul
- R01-CA-16740    Proteases in Growth Control and Malignant Transformation  
Quigley                      Downstate Medical Center

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R01-CA-19144	Membrane Changes Caused by Tumor Virus Transformation Buck	Wistar Institute of Anatomy and Biology
R01-CA-20026	Immunospecific Glycolipids of Normal and Cancer Cells Hakomori	Fred Hutchinson Cancer Research Center
R01-CA-20421	Mutants Altered in Glycosylation of Membrane Proteins Krag	Johns Hopkins University
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R01-CA-27062 Galbraith	Immunobiology of Membrane: Serum Protein Interactions Medical University of South Carolina
R01-CA-27285 Peterson	Plasma Membranes and Control of Cell Growth Boston University
R01-CA-27389 Singer	Cell Surface Fibrous Proteins and Control of Metastasis Institute for Medical Research of Bennington
R01-CA-27441 Brysk	Cell Surface Changes in Epidermal Differentiation University of Texas Med. Br., Galveston
R01-CA-27460 Ruoslahti	Alpha-Fetoprotein: Structure and Function La Jolla Cancer Research Foundation
R01-CA-27648 Johnson	Tumorigenesis and a Cell Surface Growth Inhibitor Kansas State University
R01-CA-27755 Culp	Fibronectin: Proteoglycan Binding in Adhesion Sites Case Western Reserve University

R01-CA-28101	Glycosaminoglycans in Normal and Malignant Cells Ruoslahti	City of Hope National Medical Center
R01-CA-28287	Driving Forces for Nutrient Transport in Tumor Cells Smith	University of Texas Hlth. Sci. Ctr., San Antonio
R01-CA-28548	Developing Immunological Probes for Gap Junctions Johnson	University of Minnesota of Minneapolis-St. Paul
R01-CA-28685	Phosphorylation Events and Transformed Cell Membranes Gordon	University of Colorado Hlth. Sciences Ctr.
R01-CA-28867	Cell Surface Studies of Metastatic Melanoma Nicolson	University of Texas System Cancer Center
R01-CA-29172	Structure and Function of the 170K Glycoprotein Carter	Fred Hutchinson Cancer Research Center
R01-CA-29271	Pathobiology of Pancreatic Acinar Cell Carcinoma Rao	University of Pittsburgh
R01-CA-29330	Models for Cellular Adhesion and Contact Sensation Brewer	University of Southern California
R01-CA-29397	Basement Membrane Degradation by Tumor Cells Jones	Children's Hospital of Los Angeles
R01-CA-29571	Malignant Cell Variants of Lymphosarcoma Nicolson	University of Texas System Cancer Center
R23-CA-29814	Noncytolytic Extraction of Tumor Antigens with Butanol Le Grue	University of Texas Hlth. Sci. Ctr., Houston
R01-CA-29995	Pathobiology of Malignant Cell Basement Membrane Furcht	University of Minnesota of Minneapolis-St. Paul
R23-CA-30030	Sodium Ion Fluxes and Mitogenic Signaling Smith	University of Alabama in Birmingham
R01-CA-30117	Epithelial-Mesenchymal Interaction in Endocrine Tissues Reid	Yeshiva University
R23-CA-30129	SRC Gene Expression--Modified Junctional Permeability Atkinson	University of Minnesota of Minneapolis-St. Paul
R01-CA-30192	Modification of Tumor Cell Behavior by Liposomes Poste	University of Pennsylvania
R01-CA-30289	Human Epithelial Cells-Extracellular Matrix Interactions Vlodavsky	Hebrew University of Jerusalem
R01-CA-30381	Study of the Plasma Membrane Matrix of Lymphoid Cells Mescher	Harvard University

R01-CA-30397 Steiner	Characterization of Novel Fucosides University of Kentucky
R01-CA-30471 Shaper	Role of Gal-Transferase in Cell Adhesion and Neoplasia Johns Hopkins University
R01-CA-30538 Jamieson	Interactions of Platelets and Tumor Cells American National Red Cross
R01-CA-30645 Stanley	Glycosylation Mutants of Animal Cells Yeshiva University
R01-CA-31103 Hixson	Molecular Determinants of Multicellular Organization University of Texas System Cancer Center
R01-CA-31182 Carraway	Ecto 5'-Nucleotidase as a Cell Surface Reporter University of Miami
R01-CA-31277 Isselbacher	Galactosyltransferase in Malignancy and Transformation Massachusetts General Hospital
R23-CA-31314 Crain	Composition and Function of Hepatoma Cell Membranes University of Connecticut, Storrs
R23-CA-31530 Grabel	Intercellular Adhesion of Teratocarcinoma Stem Cells University of California, San Francisco
R01-CA-31531 Johnson	Monoclonal Antibodies to a Cell Surface Glycopeptide Kansas State University
R23-CA-31573 Pantazis	Metastases Induction in Embryonal Carcinoma Cells Medical College of Georgia
R01-CA-31761 Taub	Cell Surface Sugars in Pathogenesis and Therapy of CML Columbia University
R01-CA-31767 Schwarz	Manipulation of the Differentiated State by Oncogenesis Jackson Laboratory
R01-CA-31877 Biswas	Mammalian Collagenase in Tumor Invasion Tufts University
R01-CA-32311 Damsky	Cytoskeleton-Membrane Interaction--Antisera Induced Wistar Institute of Anatomy and Biology
R01-CA-32322 Montgomery	(Na <sup>+</sup> + K <sup>+</sup> )-ATPase of Normal and Tumor Cells University of Iowa
R01-CA-32829 Izzard	Defective Adhesion in Transformed Cells State University of New York at Albany
R01-CA-32927 Levinson	Anion Transport in Ehrlich Carcinoma Cells University of Texas Hlth. Sci. Ctr., San Antonio



R01-CA-22409 Deuel	Enzyme Regulation in Normal and Neoplastic Cells Jewish Hospital of St. Louis
R01-CA-22717 Shane	Regulation of Folate Polygamma-Glutamate Synthesis Johns Hopkins University
R01-CA-23391 Shoji	Cyclic GMP System in Cell Proliferation Emory University
R01-CA-23533 Doellgast	Tumor Immunology: Phosphatase Isoenzymes Wake Forest University
R01-CA-25005 Greengard	The Regulation of Mammalian Enzyme Synthesis Mount Sinai School of Medicine
R01-CA-26033 Nathan	White Blood Cell Function in Hematologic Disorders Children's Hospital Medical Center
R01-CA-26102 Criss	Protein Kinase System in Rapidly Growing Hepatomas Howard University
R01-CA-26470 Johnson	Regulation of Dihydrofolate Reductase Gene Expression Ohio State University
R01-CA-26546 Canellakis	Regulation of Enzyme Activity in Normal and Tumor Cells Yale University
R01-CA-27073 Dunn	Cellular Enzyme Changes in Childhood Leukemia Virginia Commonwealth University
R23-CA-27500 Takemoto	The Control of Cyclic GMP in Human Lymphocytes Kansas State University
R01-CA-27572 Cory	Ribonucleotide Reductase of Tumor Cells University of South Florida
R01-CA-27674 Evans	Control of Pyrimidine Biosynthesis in Mammalian Cells Wayne State University
R01-CA-27674 Evans	Control of Pyrimidine Biosynthesis in Mammalian Cells Wayne State University
R01-CA-28111 Taub	Hormonal Regulation of Kidney Epithelial Cell Growth University of California, San Diego
R01-CA-28376 Silber	Nucleotide Biosynthesis and Degradation New York University
R01-CA-28725 Schuster	Asparagine Biosynthesis in Normal and Tumor Cells University of Nebraska, Lincoln
R01-CA-28781 Adair	Dolichyl Phosphate Biosynthesis in Tumor Cells University of South Florida

R23-CA-28973 Epstein	A Cell Culture Model for Regulation of Tumor Cell Growth Children's Hospital Medical Center
R01-CA-29048 Coffino	Ornithine Decarboxylase Deficient Animal Cell Mutants University of California, San Francisco
R01-CA-29307 Baker	Control of Protease Action on Human Cells University of Kansas, Lawrence
R01-CA-30965 Neifeld	Neuroendocrine Responsiveness of Melanoma Virginia Commonwealth University
R01-CA-32022 Anderson	Characterization of a Novel Lactate Dehydrogenase Roswell Park Memorial Institute
R01-CA-32369 Da Costa	Folate Binders in Hematopoiesis and Cell Replication State University of New York
R01-CA-32964 Weber	Glycolysis in RSV-Transformed Cells University of Illinois, Urbana-Champaign

#### PEPTIDE HORMONES

R01-CA-07535 MacLeod	Control of Pituitary Gland and Pituitary Tumor Hormones University of Virginia, Charlottesville
R01-CA-11685 Orth	Tumor Cell Synthesis and Secretion of Peptide Hormones Vanderbilt University
R01-CA-16417 Ramachandran	Pituitary Hormones in Normal and Neoplastic Growth University of California, San Francisco
R01-CA-21534 Cox	Mechanism of Ectopic Hormone Synthesis by Tumor Cells Iowa State University of Science & Tech.
R01-CA-22394 Thompson	Hormonal Control of Cell Proliferation University of South Carolina at Columbia
R01-CA-23185 Kourides	Regulation of Alpha and Beta Subunits of TSH Sloan Kettering Institute for Cancer Research
R01-CA-23603 Ascoli	Gonadotropin Actions in Leydig Tumor Cells Vanderbilt University
R01-CA-24050 Richardson	ACTH Secretion by Pituitary Tumor Cells in Culture Harvard University
R01-CA-24604 Surks	Triiodothyronine Receptors and Nonthyroidal Disease Montefiore Hospital and Medical Center
R01-CA-28218 Biswas	Hormone Production by Pituitary Tumor Cells Harvard University



ROI-CA-29467 Vanaman	The Biochemistry of Cellular Transformation Duke University
ROI-CA-29808 Iyengar	Molecular Mechanism of Desensitization Baylor College of Medicine
ROI-CA-30253 Mason	A Study of Tropic Hormone Action in Carcinoma Cells University of Texas Hlth. Sci. Ctr., Dallas
ROI-CA-30388 Golde	Humoral Regulation of Normal and Malignant Hemopoiesis University of California, Los Angeles
ROI-CA-30393 Fuller	Endocrine Regulation of Melanoma Cell Differentiation Texas Tech. University
ROI-CA-33030 Rosenfeld	Neuroendocrine Peptide Switching Events in Cancer University of California, San Diego
ROI-CA-33213 Malarkey	Prolactinomas: In Vivo and In Vitro Studies Ohio State University
ROI-CA-33336 Stouffer	Gonadotropin Receptors in Melanoma and Ovarian Cancer University of Arizona
ROI-CA-33389 Johnson	Hormonal Effects on Gastric and Hepatobiliary Cancers St. Louis University

#### STEROIDS

ROI-CA-02071 Levitz	Estrogen Metabolism and Action in Pregnancy and Cancer New York University
ROI-CA-02758 Kandutsch	Steroid Metabolism in Tumors and Normal Tissues Jackson Laboratory
ROI-CA-08315 Melnykovich	Steroid Induced Changes in Cultured Malignant Cells University of Kansas
ROI-CA-13103 Mawhinney	Metabolism and Inhibition of Prostatic Neoplasia West Virginia University
ROI-CA-13410 Sonnenschein	Mechanism of Hormone Action of Target Cells in Culture Tufts University
ROI-CA-15776 Leav	Prostatic Differentiation and Sex Hormone Metabolism Tufts University
ROI-CA-16091 Sharma	Biochemical Control of Adrenocortical Carcinoma Cells University of Tenn. Center Health Science
ROI-CA-17323 Munck	Glucocorticoid-Resistant Leukemic Lymphocytes Dartmouth College

R01-CA-19907 Harrison	Physiology of Pituitary Cell Glucocorticoid Binding Vanderbilt University
R01-CA-23248 Hymer	Prolactin Cell Function in Breast Cancer Pennsylvania State University, Univ. Park
R01-CA-23921 Colas	Biochemical and Clinical Aspects of Steroid Receptors University of Wisconsin, Madison
R01-CA-24347 Thompson	Hormone Effects on Proliferation of Malignant Thymocytes University of South Carolina at Columbia
R01-CA-25365 Gerschenson	Hormonal Regulation of Cultured Endometrial Cells University of Colorado Hlth. Sciences Ctr.
R01-CA-26112 Clark	Effect of Estrogen on Normal and Abnormal Cell Growth Baylor College of Medicine
R01-CA-26617 Sirbasku	Estrogen Mediated Pituitary Tumor Cell Growth University of Texas Hlth. Sci. Ctr., Houston
R01-CA-26638 Moyle	Cyclic AMP: Role in Adrenal Tumor Steroidogenesis Rutgers Medical School
R01-CA-27702 Siiteri	Sex Hormones, Cancer and the Immune System University of California, San Francisco
R01-CA-27828 Eisenfeld	Pituitary Steroid Receptors, Estrogens, and Adenomas Yale University
R23-CA-28580 Chaudhuri	Therapeutic Implications of Hormone in Nevi and Melanoma University of Illinois Medical Center .
R01-CA-29324 Hilf	Interactions of Estrogen Receptor with Chromatin University of Rochester
R01-CA-29497 Hall	An Adrenal Tumor: Cytochrome P-450 and Steroidogenesis Worcester Fdn. for Exper. Biology
R01-CA-30116 Shain	Hormonal Regulation of Prostate Cancer Cell Behavior Southwest Foundation for Research & Educ.
R01-CA-30380 Young	Glucocorticoid Suppression of Transformed Cell Growth Boston University
R01-CA-31835 Kallos	Actions of Estrogens and Antiestrogens in the Nucleus Columbia University
R01-CA-32178 Sherman	Hormonal Control of Fibrinolysis in Gynecologic Tissue Sloan Kettering Institute for Cancer Research
R01-CA-32226 Harmon	Steroid Resistance in Human Leukemic Cells U.S. Uniformed Services Univ. of Hlth. Sci.

## MEMBRANEOUS ORGANELLES

- |                           |  |
|---------------------------|--|
| R01-CA-06576<br>Novikoff  | Biological Effects of a Placental Protease Inhibitor<br>Yeshiva University               |
| R01-CA-08964<br>Racker    | Energy Metabolism in Normal and Tumor Cells<br>Cornell University                        |
| R01-CA-10951<br>Pedersen  | Control of Enzymatic Phosphate Transfer in Mitochondria<br>Johns Hopkins University      |
| R01-CA-12312<br>Clayton   | Mitochondrial Gene Expression in Malignant Cells<br>Stanford University                  |
| R01-CA-12858<br>Stahl     | Lysosome Biogenesis: Normal and Tumor Cells<br>Washington University                     |
| R01-CA-16527<br>Robberson | Regulation of Mitochondrial DNA Replication<br>University of Texas System Cancer Center  |
| R01-CA-20454<br>Chan      | Adenine Nucleotide Translocation in Tumor Mitochondria<br>Syracuse University, Syracuse  |
| R01-CA-25360<br>Lehninger | Respiration-Coupled Transport Processes in Tumor Cells<br>Johns Hopkins University       |
| R01-CA-25633<br>Mangel    | A New Assay for Transformed Cells<br>University of Illinois, Urbana-Champaign            |
| R01-CA-27117<br>Knowles   | A Study of Membrane Bound ATPases of Human Tumors<br>University of California, San Diego |
| R01-CA-28677<br>Coleman   | Transport in Cholesterol-Rich Tumor Mitochondria<br>New York University                  |
| R01-CA-31265<br>O'Neal    | Cation Translocation in Normal and Tumor Cells<br>University of Oklahoma, Norman         |
| R01-CA-32742<br>Pedersen  | Glucose Catabolism in Neoplastic Tissues<br>Johns Hopkins University                     |

## RIBOSOMES AND POLYRIBOSOMES

- |                          |  |
|--------------------------|--|
| R01-CA-04186<br>Rich     | Molecular Structure of Nucleic Acids and Proteins<br>Massachusetts Institute of Technology |
| R01-CA-08416<br>Penman   | Cell Architecture and Macromolecular Metabolism<br>Massachusetts Institute of Technology   |
| R01-CA-16608<br>Hardesty | Translation Control in Reticulocytes and Leukemic Cells<br>University of Texas, Austin     |

R01-CA-21663	Intermediary Metabolism in Animals and in Man
Henshaw	University of Rochester
R01-CA-27655	Gene Amplification in Carcinogenesis
Miller	Columbia University
R01-CA-28513	Structure of Hypomethylated Tumor 5.8S Ribosomal RNA
Lightfoot	Eastern Washington University

#### M-RNA

R01-CA-12550	RNA Synthesis and Transport in Mammalian Cells
Martin	University of Chicago
R01-CA-17287	Aspects of Control in Mammalian Gene Expression
Stark	Stanford University
R01-CA-18065	Biogenesis of Messenger RNA in Animal Cells
Edmonds	University of Pittsburgh
R01-CA-19535	Structure of Nuclear RNA and Messenger RNA Formation
Molloy	University of Delaware
R01-CA-20124	Messenger RNA of Normal and Malignant Human Cells
Saunders	University of Texas System Cancer Center
R01-CA-22302	Analysis of Gene Regulation by Nuclear Transplantation
Lucas	State University New York, Stony Brook
R01-CA-23226	Gene Expression in Regenerating and Neoplastic Livers
Fausto	Brown University
R01-CA-24165	Hemoglobin Studies in Friend Leukemia
Zucker	Papanicolaou Cancer Research Institute
R01-CA-24206	Embryonal Carcinoma Growth and Differentiation
Linney	La Jolla Cancer Research Foundation
R01-CA-24273	Expression of Globin Genes--Erythroleukemia Cells
Rovera	Wistar Institute of Anatomy and Biology
R01-CA-24635	Chromosomal Manifestations of Gene Expression
Melera	Sloan Kettering Institute for Cancer Research
R01-CA-25078	Poly(A) Polymerases from Liver and Hepatomas
Jacob	Pennsylvania State Univ. Hershey Med. Ctr.
R01-CA-26790	Phenotypic Variation and Neoplastic Progression
Peterson	Children's Hospital Med. Ctr. Northern Ca.
R01-CA-27932	Developmentally Regulated Genes in Teratocarcinoma
Solter	Wistar Institute of Anatomy and Biology

R01-CA-28555	Neoplastic Activation of Fetal Gene Expression Hoch Scripps Clinic and Research Foundation
R01-CA-30124	Role of 2-5A in Growth Arrest and Hormone Responses Stark Stanford University
R01-CA-30136	Control of Gene Expression in Erythropoiesis Curtis Wistar Institute of Anatomy and Biology
R01-CA-30151	Regulation of Albumin Synthesis by Amino Acids Ledford Medical University of South Carolina
R01-CA-31810	Control of mRNA Processing in Normal and Transformed Cells Rottman Case Western Reserve University
R01-CA-31894	Control of rRNA Synthesis by Carcinogens and Hormones Jacob Pennsylvania State Univ. Hershey Med. Ctr.

#### T-RNA

R01-CA-10922	Metabolism of Animal Cell Ribosomes Vaughan University of Pittsburgh
R01-CA-13591	Chemical Studies on Tumor Nucleic Acids Randerath Baylor College of Medicine
R01-CA-20683	Control Mechanisms in Human Tumor Cells--Small RNAs Eliceiri St. Louis University
R01-CA-20919	tRNA Q Base Biosynthesis, Effect on Cellular Expression Katze University of Tenn. Center Health Sciences
R01-CA-21245	Structure and Modification of Mammalian Transfer RNA Penhoet University of California, Berkeley
R01-CA-23536	Molecular Biology of Ethionine Carcinogenesis Borek AMC Cancer Research Center and Hospital
R01-CA-26423	Modification of tRNA/Lys Controls Cell Proliferation Ortwerth University of Missouri, Columbia
R01-CA-27235	tRNA Modification and Gene Expression in Mammalian Cells Hatfield University of California, Irvine
R01-CA-28053	Thiolated tRNAs in Rat Liver and Morris Hepatomas Wong University of Chicago
R01-CA-28395	tRNA Methylation in Normal and Neoplastic Rat Tissues Leboy University of Pennsylvania
R01-CA-31313	Expression of t-DNA Sequences in Crown Gall Tumors Thomashow Washington State University



# DNA

R01-CA-14835 Korn	DNA Polymerases in Normal and Neoplastic Human Cells Stanford University
R01-CA-15044 Manuelidis	Pathogenetic Determinants of Human CNS Tumors Yale University
R01-CA-15187 Baril	DNA Synthesis: Regulation in Normal and Cancer Cells Worcester Fdn. for Exper. Biology
R01-CA-16790 Maio	DNA Transcription Control in Normal and Cancer Cells Yeshiva University
R01-CA-17723 Meyer	Regulation of DNA Synthesis in the Novikoff Hepatoma University of Cincinnati
R01-CA-18138 Pegg	Mammalian Polyamine Metabolism Pennsylvania State Univ. Hershey Med. Ctr.
R01-CA-23365 Chang	Function of DNA Polymerases in Normal and Cancer Cells U.S. Uniformed Services Univ. of Hlth. Sci.
R01-CA-24158 Collins	DNA Synthesis in Transformed Cells Virginia Commonwealth University
R01-CA-24323 Mathews	DNA Precursor Dynamics in Animal Cells Oregon State University
R01-CA-24845 Loeb	The Fidelity of DNA Replication in Human Lymphocytes University of Washington
R01-CA-26391 Coleman	Molecular Pathology of Leukemia and Lymphoma University of Kentucky
R23-CA-30387 Albert	Deoxynucleoside Triphosphate Metabolism and DNA Synthesis Sidney Farber Cancer Institute

# GROWTH FACTORS

R01-CA-11176 Holley	Factors Required for Mammalian Cell Division Salk Institute for Biological Studies
R01-CA-14019 Folkman	Tumor Angiogenesis: A Control Point in Tumor Growth Children's Hospital Medical Center
R01-CA-17203 Tupper	Role of Serum and Cations in Malignant Cell Growth Syracuse University, Syracuse
R01-CA-17620 Smith	Growth Control in Normal and Neoplastic Cells University of Nebraska, Lincoln

R01-CA-19275	Normal and Leukemic Granulopoiesis
Dodge	Wake Forest University
R01-CA-19731	Human Tumor Culture Lines in Defined Media
Sato	University of California, San Diego
R01-CA-21763	Cartilage and Chondrosarcoma-Derived Growth Factors
Klagsbrun	Children's Hospital Medical Center
R01-CA-22410	Copper Absorption and Ceruloplasmin in Cancer
Linder	California State University, Fullerton
R01-CA-23528	Inhibitory Interactions Regulating Hematopoiesis
Broxmeyer	Sloan Kettering Institute for Cancer Research
R01-CA-24071	Receptors for Epidermal Growth Factor in Tumor Cells
Carpenter	Vanderbilt University
R01-CA-25820	Receptors and Growth Factors for Neoplastic Cells
Schlessinger	Weizmann Institute of Science
R01-CA-27113	Molecular Analysis of Platelet-Derived Growth Factor
Scher	Sidney Farber Cancer Institute
R01-CA-27217	Growth Factors and Receptors in Chemical Transformation
Moses	Mayo Foundation
R01-CA-27466	Endothelial Colony-Stimulating Activity
Quesenberry	University of Virginia, Charlottesville
R23-CA-27802	Pharmacologic Modulation of Erythroid Colony Formation
Beckman	Tulane University of Louisiana
R01-CA-28110	Nerve Growth Factor: Secretion by Cancer Cells
Young	University of Florida
R01-CA-28540	Growth and Migration of Capillary Endothelial Cells
Zetter	Children's Hospital Medical Center
R01-CA-28638	Altered Nutritional Requirements for Growth
Topp	Cold Spring Harbor Laboratory
R01-CA-28858	Biological and Synthetic Modulators of Cell Growth
Pickart	Virginia Mason Research Center
R01-CA-29101	Characterization of a Liver Specific Growth Promotor
La Brecque	University of Iowa
R01-CA-30101	Structure and Function of Platelet-Derived Growth Factor
Antoniades	Center for Blood Research
R01-CA-30479	Mononuclear Phagocyte-Derived Growth Regulating Factors
Gillespie	University of North Carolina, Chapel Hill

R01-CA-30536 Wells	New Myeloid Hemapoietins: Normal and Leukemic Marrow University of California, Los Angeles
R01-CA-31279 Haigler	Epidermal Growth Factor: Interactions with Cell Receptor University of California, Irvine
R01-CA-31529 Rossow	The Hormonal Regulation of Normal Cell Growth Jackson Laboratory
R01-CA-31615 Adamson	Growth Factors in Normal and Neoplastic Hematopoiesis University of Washington
R01-CA-31790 Fenselau	Control of Tumor-Induced Vascularization Papanicolaou Cancer Research Institute
R01-CA-31796 Lim	Effect of Glia Maturation Factors on Tumors University of Iowa
R01-CA-34162 Scher	Growth Factors and Cellular Transformation Children's Hospital of Philadelphia

#### NUCLEUS

R01-CA-12226 Paik	Protein Methylation in Neoplastic Tissues Temple University
R01-CA-12877 Langan	Function of Lysine Rich (H1) Histone Phosphorylation University of Colorado Hlth. Sciences Ctr.
R01-CA-13195 Smulson	Histone ADP-Ribosylation and Hela Cell Replication Georgetown University
R01-CA-15135 Zweidler	Histones in Cell Differentiation and Carcinogenesis Fox Chase Cancer Center
R01-CA-16910 Rowley	Chromosome Aberrations in Myeloproliferative Diseases University of Chicago
R01-CA-17782 Reeck	Tumor-Enriched Nonhistone Chromatin Proteins Kansas State University
R01-CA-18389 Hnilica	Proteins of the Cell Nucleus Vanderbilt University
R01-CA-18455 Wray	Isolated Chromosomes in Genetic and Cancer Research Baylor College of Medicine
R01-CA-21927 Maizel	Chromatin Structure of Normal and Malignant T-Cells University of Texas System Cancer Center
R01-CA-24546 Kornberg	Relation of Histones to DNA in Normal and Cancer Cells Stanford University

R01-CA-25055	Cytogenetics of Clonal Neoplasias
Hecht	Southwest Biomedical Research Institute
R01-CA-27661	Sister Chromatid Exchange in ALL
Palmer	Indiana Univ.-Purdue Univ. at Indianapolis
R01-CA-28679	Chromosomal Organization of Dihydrofolate Reductase Gene
Biedler	Sloan Kettering Institute for Cancer Research
R01-CA-29340	rDNA Distribution in Chromosomes of Neoplastic Cells
Henderson	Columbia University
R01-CA-29476	Clonal Karyotypic Evolution in Human Solid Tumors
Trent	University of Arizona
R01-CA-29617	Role of Double Minutes and HSR Markers in Tumor Cells
George	Johns Hopkins University
R01-CA-29779	DNA Replication: Chromosomes and Neoplasms
Cervinka	University of Minnesota of Minneapolis-St. Paul
R01-CA-31024	Fine Structural Chromosomal Defects in Acute Leukemia
Yunis	University of Minnesota of Minneapolis-St. Paul
R01-CA-32572	Biochemistry of Late Mitotic Events in Cultured Cells
Hodge	Medical College of Georgia
R01-CA-33314	Fine Chromosomal Defects in Non-Hodgkin's Lymphoma
Yunis	University of Minnesota of Minneapolis-St. Paul

#### CONTRACTILE ELEMENTS

R01-CA-05493	Leukopoietic Mechanisms
De Bruyn	University of Chicago
R01-CA-15544	Effect of Microtubular Proteins on Cell Surfaces
Berlin	University of Connecticut Health Center
R01-CA-22031	Trans-Membrane Control in Transformed and Normal Cells
Singer	University of California, San Diego
R01-CA-26473	ACTH Effects on the Cytoskeleton of Adrenal Tumor Cells
Mattson	Case Western Reserve University
R01-CA-26867	Invasion and Metastasis
Zeidman	University of Pennsylvania
R01-CA-28362	Levels of Metastasis Inhibition in Primary Cancers
Rapp	Roswell Park Memorial Institute
R01-CA-29985	Microtubules and Nonmicrotubular Aggregates
Weisenberg	Temple University

R01-CA-31460	Contractile Protein in Normal and Transformed Cells
Kiehart	Johns Hopkins University
R01-CA-33265	Tropomyosin and Stress Fibers: Transformed Cells
Warren	University of Miami
R01-CA-34155	Movement of Virus-Transformed Cell Cytoplasm
Albrecht	Northwestern University

#### DEVELOPMENT AND DIFFERENTIATION

R01-CA-02662	Investigations on Teratocarcinogenesis
Stevens	Jackson Laboratory
R01-CA-10095	Gene Action and Cellular Differentiation in Culture
Silagi	Cornell University Medical Center
R01-CA-13047	Control Mechanisms of Differentiation and Malignancy
Friend	Mount Sinai School of Medicine
R01-CA-13533	Ectopic Placental Proteins in Cancer
Sussman	Stanford University
R01-CA-14319	Biological Functions of Plasma Membrane Sialidase
Schengbund	Pennsylvania State Univ. Hershey Med. Ctr.
R01-CA-15222	Hepatoma Alpha-Fetoprotein: Chemistry and Metabolism
Smith	University of Vermont & St. Agric. College
R01-CA-16368	Control of Differentiation of Erythroleukemic Cells
Skoultchi	Yeshiva University
R01-CA-17389	C-Cell Hyperplasia and Medullary Carcinoma of Thyroid
Wolfe	Tufts University
R01-CA-17575	Erythroid Differentiation in Friend Leukemia Cells
Housman	Massachusetts Institute of Technology
R01-CA-18375	Hemopoietic Stem Cells and Induced Differentiation
Goldwasser	University of Chicago
R01-CA-19492	Terminal Transferase in Mammalian Hemopoietic Tissue
Coleman	University of Kentucky
R01-CA-20053	Tumor Inception and Progression
Meins	University of Illinois, Urbana-Champaign
R01-CA-21566	Antitumor Invasion Factors Derived from Cartilage
Kuettner	Rush-Presbyterian-St. Lukes Medical Ctr.
R01-CA-22227	Onco-Developmental Gene Control: Alpha-Fetoprotein
Sell	University of California, San Diego



R01-CA-22294	Quantitative Studies on Granulocyte Differentiation
Kinkade	Emory University
R01-CA-22556	Differentiation of Granulocytes and Monocytes
Metcalf	Walter and Eliza Hall Inst. Medical Res.
R01-CA-22735	Thymic Modification of Leukemogenesis
Sensenbrenner	Johns Hopkins University
R01-CA-23097	Teratocarcinoma and Onco-Embryonic Phenotype
Damjanov	Hahnemann Med. Col. & Hosp. of Philadelphia
R01-CA-23615	Molecular Basis of Differentiation and Neoplasia
Roeder	Washington University
R01-CA-24241	Differentiation in a Malignant Neural Tumor
Sandquist	University of Iowa
R01-CA-24479	Membrane Structure and Enzyme Induction
Chen	Rutgers, The State University, New Brunswick
R01-CA-24488	The Controlled Initiation of Neoplasms in Drosophila
Hanratty	University of California, Irvine
R01-CA-25098	Alpha-Fetoprotein Regulation in Fetal and Cancer Liver
Chiu	University of Vermont & St. Agric. College
R01-CA-25164	Malignant Phenotypes: Cytoplasmic and Nuclear Control
Sudilovsky	Case Western Reserve University
R23-CA-25285	Ectopic Antigens in Hepatocellular Cancer
Higgins	Sloan Kettering Institute for Cancer Research
R01-CA-25512	Modulators of Granulopoiesis from Human Cell Lines
Brennan	University of Rochester
R01-CA-25799	Conformation of Alpha-Fetoprotein Gene in Chromatin
Anderson	Purdue University
R01-CA-25966	X-Chromosome Activity in Teratocarcinoma Stem Cells
Martin	University of California, San Francisco
R01-CA-25985	Response of Phagocytic Leukocytes to Tumor Promoters
Christman	Mount Sinai School of Medicine
R01-CA-26014	Cell Interactions in Teratocarcinoma Differentiation
Rosenstrauss	Rutgers, The State University, New Brunswick
R01-CA-26038	Study of Myeloid Leukemia Using Human Leukemia Lines
Koeffler	University of California, Los Angeles
R01-CA-26105	Human Renal Cancer and Hematopoiesis
Sytkowski	Children's Hospital Medical Center

R01-CA-26656	Cell Culture Analysis of Human Epidermal Neoplasia Rheinwald	Sidney Farber Cancer Institute
R01-CA-26847	Regulation of mRNA Translation in Animal Cells Weber	University of South Florida
R01-CA-26993	Cytotoxic Factor(s) from Human Ovarian Adenocarcinoma Sheid	Downstate Medical Center
R01-CA-27287	Vaginal and Cervical Epithelial Cell Culture Model Rice	Harvard University
R01-CA-27682	New Technology for Classifying Human Leukemia Stang	University of California, Los Angeles
R01-CA-28050	Regulation of Alpha-Fetoprotein Gene Expression Tilghman	Institute for Cancer Research
R01-CA-28106	Differentiation of Teratocarcinoma Cells Levine	State University of New York, Stony Brook
R01-CA-28179	Lithium Effects on Hemopoietic Stem Cells Joyce	University of Pittsburgh
R01-CA-28228	Expansion of Hemopoietic Bone Marrow Lee	University of Washington
R23-CA-28289	Nuclear vs. Extranuclear Control of Gene Expression Moore	University of Colorado Hlth. Sciences Ctr.
R01-CA-28427	EGF and Its Receptors in Embryonic Differentiation Adamson	La Jolla Cancer Research Foundation
R23-CA-28512	Regulation of Normal and Leukemic Myeloid Stem Cells Pelus	Sloan Kettering Institute for Cancer Research
R01-CA-28656	Differentiation of Capillary Endothelial Cells Auerbach	University of Wisconsin, Madison
R01-CA-29169	Gene Expression Embryonal Carcinoma Differentiation Linney	La Jolla Clinic Research Foundation
R01-CA-29894	Differentiation in Human Teratoma Derived Cell Lines Andrews	Wistar Institute of Anatomy and Biology
R01-CA-29895	Antiproliferative Effects of Interferons Baglioni	State University of New York at Albany
R01-CA-30049	Oncofetal Gene Regulation in Hepatocarcinogenesis Papaconstantino	University of Texas Med. Br., Galveston
R01-CA-30684	Regional Differences in Tumor Growth and Development Auerbach	University of Wisconsin, Madison

R01-CA-31042 Lo	Developmental Regulation of B Globin Gene Expression University of Pennsylvania
R01-CA-31271 Rubinstein	Differentiation and Stroma-Induction in Neural Tumors University of Virginia
R01-CA-31667 Rothenberg	RNAs of Lymphoma and T Cell Differentiation Antigens Salk Institute for Biological Studies
P01-CA-31768 Marks	Leukemia Cell Systems: Induction of Differentiation Sloan Kettering Institute for Cancer Research
R01-CA-31937 Graf	Control of Melanoma Cell Differentiation: Genetic Study Cornell University Medical Center
R01-CA-31945 Lozzio	K-562: A Human Pluripotent Leukemia Stem Cell Line University of Tennessee, Knoxville
R01-CA-32186 Salser	REC-DNA Analysis of Human Hematopoietic Differentiation University of California, Los Angeles
R23-CA-32260 Krystosek	Differentiation and Malignancy in Neural Cell Culture University of Colorado Hlth. Sciences Ctr.
R01-CA-32586 Cohen	Myeloid Development in an Induced Leukemic Cell Line University of Rochester
R23-CA-32733 Abrahm	Modulation of Normal and Abnormal Human Myelopoiesis University of Pennsylvania
R01-CA-33000 Fukuda	Glycoproteins in Differentiation and Oncogenesis La Jolla Cancer Research Foundation
R01-CA-33011 Oshima	Chromatin Proteins of Embryonal Carcinoma Cells La Jolla Cancer Research Foundation
R01-CA-33065 Daynes	Immunobiology of UVL-Induced Tumors University of Utah
R01-CA-33563 Taylor	Control of Albumin Synthesis in Liver and Hepatomas Pennsylvania State Univ. Hershey Med. Ctr.
R01-CA-33579 Green	Growth and Differentiated Function of Keratinocytes Harvard University
R01-CA-33946 Oshima	Teratocarcinoma Cytoskeletal Proteins La Jolla Cancer Research Foundation

#### CELL GROWTH, CELL DIVISION

R01-CA-06663 Lieberman	Repair of Chromatin by 3-Methyladenine N-Glycosylase University of Pittsburgh
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R01-CA-08373 Baserga	Study of Factors Controlling Cellular Proliferation Temple University
R01-CA-15062 Ahmed	Studies of Normal and Neoplastic Prostate University of Minnesota of Minneapolis-St. Paul
R01-CA-15141 O'Neill	Control of Nuclear Events in Normal and Neoplastic Cells University of Utah
R01-CA-15305 Ham	Effect of Malignancy on Cell Growth Requirements University of Colorado at Boulder
R01-CA-15813 Baker	Lipid Transport and Metabolism in Cancer-HCST Systems University of California, Los Angeles
R01-CA-16463 Surks	Triiodothyronine Receptors and Thyrotroph Neoplasia Montefiore Hospital and Medical Center
R01-CA-16816 Moses	Mechanism of Chemical Carcinogenesis In Vitro Mayo Foundation
R01-CA-21359 Bertram	Cell Interactions During Malignant Transformation Roswell Park Memorial Institute
R01-CA-22042 Stiles	Molecular Analysis of Progression Through G1 Sidney Farber Cancer Institute
R01-CA-23022 Brinkley	Mitosis in Normal and Neoplastic Cells Baylor College of Medicine
R01-CA-24193 Pledger	Regulation of Mammalian Cell Cycle University of North Carolina, Chapel Hill
R01-CA-24385 Mastro	Effects of Phorbol Esters on Lymphocyte Stimulation Pennsylvania State University, Univ. Park
R01-CA-26070 Basilico	Control of Cycle Progression in Animal Cells New York University
R01-CA-26081 Varga	Cell Cycle Dependence on Cell Surface Receptors Yale University
R01-CA-27151 Reiter	Regulation of Cell Replication by Thymidine University of Illinois Medical Center
R01-CA-27399 Sisken	Regulation of Mitosis in Normal and Transformed Cells University of Kentucky
R01-CA-27544 Rao	Purification and Characterization of Mitotic Factors University of Texas System Cancer Center
R01-CA-27562 Litt	Regulation of Transfer RNA Levels in Mammalian Cells Oregon Health Sciences University

R01-CA-27564 Hoffman	Methionine Dependence--A Metabolic Marker in Cancer University of California, San Diego
R01-CA-27809 Sauer	Pathways of Energy Metabolism in Malignancy In Vivo Mary Imogene Bassett Hospital
R01-CA-28140 Tryfiates	A Novel Vitamin B6 Metabolite in Hepatomas West Virginia University
R01-CA-28238 Vogel	Effects of Mitogens on Normal and Neoplastic Cells University of Washington
R01-CA-28240 Scott	Pathology in Cell Cycle Control of Differentiation Mayo Foundation
R23-CA-28329 Keng	Phase Specific DNA Repair in Irradiated Tumor Cells University of Rochester
R01-CA-28519 Rosenblum	Characterization of Cells and Clones from Human Brain University of California, San Francisco
R01-CA-28760 Hauschka	Anticoagulants, Vitamin K, and Tumor Cell Growth Children's Hospital Medical Center
R01-CA-28803 Rapaport	The Role of AP4A in Malignant Transformation Boston University
R01-CA-30083 Eisenstein	Aortic Growth Inhibitors Mount Sinai Medical Center
R01-CA-31053 Vogelstein	Mitotic Inducing Protein (S) from Mammalian Cells Johns Hopkins University
R01-CA-32094 Taetle	Humoral Control of Leukemic Blast Proliferation University of California, San Diego
R01-CA-32172 Hoffman	Regulation and Inhibition of Polyamine Metabolism University of Louisville
R01-CA-32952 Ross	Phosphotyrosine and the Control of Cell Growth Wistar Institute of Anatomy and Biology

#### SOMATIC CELL GENETICS

R01-CA-12130 Harris	Cytoplasmic Inheritance in Normal and Tumor Cells University of California, Berkeley
R01-CA-16631 Meiss	Isolation and Analysis of DNA Mutants of BHK Cells New York University
R01-CA-16754 Littlefield	Hybridization, DNA Function, Mutation in Cell Culture Johns Hopkins University



R01-CA-19401	Genetic Analysis of Human Malignancy
Stanbridge	University of California, Irvine
R01-CA-20741	Biology of Human Fibrosarcoma
Croce	Wistar Institute of Anatomy and Biology
P01-CA-20810	Somatic Cell Genetics in Cancer
Puck	University of Colorado Hlth. Sciences Ctr.
R01-CA-21054	Genetic Analysis of Malignant Transformation
Shin	Yeshiva University
R01-CA-24828	Genetic Analysis of Tumorigenesis
Sager	Sidney Farber Cancer Institute
R01-CA-25342	Complement of Sister Chromatid Exchange to Cell Hybrids
Siniscalco	Sloan Kettering Institute for Cancer Research
R01-CA-27607	Coordinated Control of Mammalian Gene Expression
Lee	University of Southern California
R01-CA-27712	Gene Expression During Mammalian Development
Croce	Wistar Institute of Anatomy and Biology
R01-CA-27713	Gene Expression During Mammalian Development
Illmensee	University of Geneva
R01-CA-28559	Study of Malignant Transformation: A Genetic Analysis
Athwal	University of Medicine & Dentistry of NJ
R01-CA-30643	Genetic Bases for the Transformed Phenotype
Ozer	Hunter College
R01-CA-30938	Structural and Functional Analysis of Cloned MHC Gene
Weissman	Yale University
R01-CA-31553	Cytogenetics and Molecular Biology of Human Neuroblastoma
Biedler	Sloan Kettering Institute for Cancer Research
R01-CA-31649	Transformation Proteins of Non-Virally Induced Tumors
Weinberg	Massachusetts Institute of Technology
R01-CA-31777	BUDR Dependence, Malignancy, and Differentiation
Davidson	University of Illinois Medical Center
R01-CA-31995	Retroviral Oncogenesis: Analysis of Cellular Homologues
Sheiness	Louisiana State Univ. Med. Ctr., New Orleans
R01-CA-33108	Cell Transforming Genes of T- and B-Lymphocyte Neoplasms
Lane	Sidney Farber Cancer Institute

# INHERITANCE OF NEOPLASMS

- R23-CA-28963 Genetic Basis of SJL/J Murine Reticulum Cell Sarcoma  
Bubbers University of California, Los Angeles
- R01-CA-29078 Cellular Origins of Rat Hepatic Preneoplasias  
Iannaccone Northwestern University
- R01-CA-32832 Cytogenetics of Familial Medullary Thyroid Carcinoma  
Wurster-Hill Dartmouth College
- R01-CA-33093 Recombinant Inbred Mouse Strains and Cancer  
Taylor Jackson Laboratory
- R01-CA-33383 Transforming Genes of Benign and Malignant Colon Tumors  
Augenlicht Sloan Kettering Institute for Cancer Research
- R23-CA-33947 Analysis of Genetic Heterogeneity in Fanconi Anemia  
Auerbach Rockefeller University

## PLASMIDS, VIRUSES

- R01-CA-11526 Tumor-Inducing Substance of Agrobacterium Tumefaciens  
Kado University of California, Davis
- R01-CA-13015 Molecular Basis of Crown Gall Tumorigenesis  
Nester University of Washington
- R01-CA-18604 The Mechanism of Tumorigenesis by A. Tumefaciens  
Matthysse University of North Carolina, Chapel Hill
- R01-CA-19402 Molecular Genetics of Agrobacterium Plasmids  
Farrand Loyola University Medical Center
- R01-CA-26963 Molecular Regulation of Crown Gall Tumor Growth  
Chang University of Wisconsin, Parkside
- R01-CA-28946 Transfection by Endogenous Human Transforming Genes  
Cooper Sidney Farber Cancer Institute
- R01-CA-29474 Cytology, Biochemistry of Viral-Specific Proteins  
Buchanan Massachusetts Institute of Technology
- R01-CA-29477 Analysis of Malignancy by Gene Transfer  
Kucher Princeton University
- R01-CA-34171 Analysis of Malignancy by Gene Transfer  
Kucher University of Illinois Medical Center

# IN VIVO AND IN VITRO TUMOR LINES

R01-CA-11683 Kaplan	Coenzymes and Nucleic Acids Metabolism University of California, San Diego
R01-CA-17229 Russell	Keloids: An In Vitro Model of Tumor Growth Regulation Meharry Medical College
R01-CA-24145 Beamer	Ovarian Tumors in Young Mice Jackson Laboratory
R01-CA-28668 Gehrke	Biologic Markers for Melanoma University of Missouri, Columbia
R01-CA-29440 Mehard	Biochemical Identification of Organ Specific Melanoma University of California, San Francisco
R01-CA-29550 Varani	Tumor Cell With Varying Degrees of Malignancy University of Michigan
R01-CA-30082 Nesbitt	Genetics and Development of Teratocarcinoma Cells University of California, San Diego
R01-CA-30621 Epstein	Biology and Immunobiochemistry of Hematopoietic Tumors Northwestern University
R01-CA-32134 Stackpole	B16 Melanoma Metastasis Model System New York Medical College
R01-CA-32318 Civin	Antigenic Analysis of Hematopoiesis Johns Hopkins University
R01-CA-32412 McKeehan	Androgen-Responsive Prostate Epithelial Cells W. Alton Jones Cell Science Center

## DIFFICULT-TO-CLASSIFY

R01-CA-09247 Philips	Partial Subsidy for the Journal of Cancer Research American Association for Cancer Research
R01-CA-22062 Nimberg	A Bone Resorptive Protein from Cancer Ascites Fluid Boston University
R01-CA-25298 Clark	Biology of Human Cutaneous Malignant Melanoma University of Pennsylvania
R01-CA-27120 Ts'o	Interferon System: Action, Induction and Regulation Johns Hopkins University
R01-CA-28571 Erickson	Recognition of Patterns in Cancer-Related Sequences Rockefeller University

R01-CA-29551      Complement Mediated Tumor Cell Chemotaxis  
Ward                      University of Michigan

PROGRAM PROJECTS

P01-CA-10893      Cancer Research Center  
Busch                      Baylor College of Medicine

P01-CA-12923      A Correlated Study on the Biology of Neoplasia  
Baserga                      Temple University

P01-CA-14454      The Plasma Membrane in Normal and Cancer Cells  
Racker                      Cornell University

P01-CA-15823      Program in Developmental Biology of Cancer  
Pierce                      University of Colorado Hlth. Sciences Ctr.

P01-CA-19265      Cancer Biology Research Center  
Ullmann                      University of Chicago

P01-CA-21901      Studies of Normal and Malignant Cell Membranes  
Roseman                      Johns Hopkins University

P01-CA-22376      Control of Gene Expression: Normal and Neoplastic  
Feigelson                      Columbia University

P01-CA-23052      Program Project on Athymic Mice and Human Tumors  
Kaplan                      University of California, San Diego

P01-CA-23052      Program Project on Athymic Mice and Human Tumors  
Kaplan                      University of California, San Diego

P01-CA-23076      Regulatory Mechanisms in Tumor Biology  
Mueller                      University of Wisconsin, Madison

P01-CA-25845      Pathobiology of Small Cell Carcinoma of the Lung  
Sorenson                      Dartmouth College

P01-CA-25875      Cell Differentiation and Cancer  
Croce                      Wistar Institute of Anatomy and Biology

P01-CA-26712      Glycoproteins, the Cytoskeleton and Cancer  
Hynes                      Massachusetts Institute of Technology

P01-CA-28853      Comparative Study of Primary and Metastatic Human Tumor  
Holyoke                      Roswell Park Memorial Institute

P01-CA-28896      Cell-Matrix Interactions in Neoplasia and Development  
Ruoslahti                      La Jolla Cancer Research Foundation

P01-CA-29545      Interferon, Differentiation and Oncogenesis  
Carter                      Hahnemann Med. Col. & Hosp. of Philadelphia

P01-CA-29569    Gene Organization and Expression in Eukaryotes  
          Watson                    Cold Spring Harbor Laboratory

P01-CA-32737    Medical Oncology  
          Golde                    University of California, Los Angeles

P01-CA-34017    Growth and Cell Control Processes  
          Green                    Harvard University

#### CONFERENCES

R13-CA-15961    Seminars and Workshops in Techniques of Cancer Research  
          King                    University of Colorado Hlth. Sciences Ctr.

R13-CA-16244    Support for Cancer Research Center Courses  
          Grodzicker              Cold Spring Harbor Laboratory

R13-CA-30706                      University of Washington  
          Stamatoyann

R13-CA-31782    Fourth International Workshop on Chromosomes in Leukemia  
          Rowley                    University of Chicago

R13-CA-33424    Gordon Research Conference on Cancer, 1982  
          Black                    Gordon Research Conferences



# CONTRACT RESEARCH SUMMARY

Title: Morris Hepatoma Resource Program

Principal Investigator:  
Performing Organization:  
City and State:

Dr. Wayne E. Criss  
Howard University College of Medicine  
Washington, D.C.

Contract Number: NCI-CB-14345-39

Starting Date: 7/1/81

Expiration Date: 6/30/84

Goal: To maintain eleven Morris hepatomas representative of the spectrum of rapidly- to very slow-growing-tumors in stock rats and provide them on request to laboratories for research purposes.

Approach: The hepatomas will be propagated by serial transplantation in rats and periodically monitored by enzyme profiles and assay of specific metabolites to assure stability of each line. Requests for any of the hepatomas will be filled, depending on availability, by injecting tumor tissue into host rats purchased by the requestor and then shipping them to his/her laboratory by air freight.

Progress: During the first year of this contract 2482 rats were either supplied to investigators on request or used in the contract facility to maintain tumor stock.

Fast growing	<u>Actual Use</u>	Intermediate Growing	<u>Actual Use</u>
5123Tc	123	7800	386
7777	368	5123D	129
3924A	842		
44	186	Slow Growing	
8999	74	9618A	119
		7787	60
		16	88
		20	14
			<u>2389</u> tumor bearing rats
		Controls (Non-tumor bearing)	93

Tumor bearing rats were sent to 45 different investigators, 40 in the U.S., 4 in Canada and 1 in the Netherlands.

The contractor has initiated an animal health surveillance program to control pin worm infection identified in Buffalo rats from one supplier. He has also negotiated an agreement to purchase rats from an additional animal facility through the National Institutes of Health.

Significance to Cancer Research: Each of these hepatomas has specific characteristics that make it the tumor of choice for certain research projects. A number of NCI grants in the areas of enzymology, intermediary metabolism and molecular biology depend upon this liver tumor system.

Project Officer: Dr. Colette Freeman  
Program: Tumor Biology Section  
FY 82 Funds: \$176,920



## IMMUNOLOGY PROGRAM

The role of the Immunology Program of the National Cancer Institute is to support studies which contribute to an understanding of the role of the immune system in the development, growth and spread of tumors. The specific areas of investigation supported by the Program include:

- ° The synthesis and structure of myeloma proteins in animals and man.
- ° The synthesis, structure, and function of antibodies capable of reacting with tumor cells, agents which induce tumors, and agents used in the treatment of tumors.
- ° The synthesis, structure, and function of humoral factors other than antibody which participate in, activate and/or regulate the immune response to tumors. This would include complement, interferon, lymphokines, lymphoid cell growth factors, helper factors, suppressor factors, etc., as they are involved in immune responses to tumors.
- ° The immunobiology of lymphocytes which participate in antitumor responses including their development, heterogeneity, interactions, and functions.
- ° The immunobiology of monocytes and macrophages which participate in antitumor responses including their development, heterogeneity, interactions, and functions.
- ° The identification, isolation, and characterization of cell surface determinants of lymphocytes and macrophages which are involved in the responses of these cells to tumors.
- ° The identification, isolation, and characterization of cell surface determinants on tumor cells which serve as target antigens for the immune response.
- ° The immunobiology of malignancies of the immune system (lymphomas and leukemias) including studies of immunologic markers for the classification and characterization of neoplastic cells and their normal counterparts.
- ° Immunobiology of sarcomas, carcinomas, and melanomas including studies of immunologic markers for the classification and characterization of tumor cells and their normal counterparts.
- ° Immune surveillance against the development of tumors of various origins by all immune mechanisms (e.g. T cell immunity, macrophage reactivity, natural killer cell activity).
- ° Immunopathology studies on the host-tumor interaction.

- ° Immune status of tumor-bearing animals and man including studies on immunostimulation, immunosuppression, and the effects of disease course on immune function.
- ° Bone marrow transplantation (BMT) in man and animals as a treatment for cancer when the emphasis is on understanding how BMT affects or is affected by the immune system.
- ° Immunotherapy in animal models including studies on specific and non-specific stimulation of the immune system using natural and synthetic agents when the emphasis is on understanding how the therapy affects or is affected by the immune system.
- ° Immunotherapy including preclinical and clinical protocols where the main emphasis is upon the study of immune parameters, immune mechanisms, and other immunologic concerns rather than upon a therapeutic result. Included are studies on specific and nonspecific stimulation of the immune system using natural and synthetic agents.

Immunology, the study of the body's defense against infection and disease, including the role of the immune system against cancer, has been an area of intense investigation over the last two decades. The immune system is extremely complex but basic research on its development, the interaction and functions of its component parts and how these functions are regulated is continually shedding new light on this system. It is clear that research on the immune system is important to the understanding of cancer and its treatment in many ways: a) how the normal immune system reacts to tumor cells, i.e. immune surveillance; b) how a stimulated immune system assists the body in eliminating cancer cells or controlling tumor growth, i.e. immune intervention; and c) how our knowledge of the immune system can be used to generate diagnostic procedures and treatment modalities. Also, some cancers actually involve cells of the immune system itself, i.e. the leukemias and lymphomas. The Immunology Program supports three major areas of research: (1) basic immunology (2) tumor immunology and (3) mechanisms of immunologic intervention.

The recent development of hybridoma technology has had a powerful impact on research in immunology; in fact, the majority of grants supported by the Immunology Program involve the use of monoclonal antibodies in some aspect of the research. The report that follows is intended to emphasize the use and, therefore, the impact of this technology on the three thrusts of research supported by this Program. Since the Immunology Program supports nearly 500 research grants, this report serves to reflect and highlight a certain portion of this research and should not be considered comprehensive.

### Basic Immunology

Studies of antigens on the surface of cells of the immune system, their biochemical characterization and role in the differentiation and regulation of immune function continue to develop at a rapid pace. The use of hybridoma technology in the preparation of hybrids secreting monoclonal antibodies capable

## Tumor Immunology

Tumor immunology encompasses the study of interactions between tumor cells and the immune system. Dr. Pellegrino has been studying the expression of Ia-like antigens on tumor cells as they may reflect stages of differentiation. Using monoclonal antibodies to identify Ia-like antigens, Dr. Pellegrino has found these antigens on normal tissues derived from endoderm, mesoderm and ectoderm (11). The expression of these antigens may be related to tumor cell transformation since immunofluorescent staining of a variety of explanted human tumors has shown that these tumors may acquire Ia-like antigens.

Currently, the most studied anti-tumor response of the immune system is lymphocyte-mediated cytotoxicity. The specificity of this response, if not through antibody, is through antigen recognition. Dr. Mukherji has utilized hybridoma technology to develop hybrids which were capable of killing human melanoma cells in vitro. He fused human lymphocytes, sensitized in vitro to two human melanoma cell lines in the presence of exogenous IL-2, to a mouse myeloma cell line. Fifteen hybrids were demonstrated to be effective in killing one of the original sensitizing melanoma cell lines (12).

A current thrust in tumor immunology is the identification of tumor-associated antigens. The use of hybridoma technology has had a significant impact in this search. Tumor-associated antigens on leukemias and lymphomas were identified in a previous report and will not be reported here. Dr. Seeger has generated a monoclonal antibody which has restricted specificity for neural cells (13). It reacts with human neuroblastoma, glioma, and melanoma. It also reacts strongly with fetal brain, less with adult brain and weakly with cultured fibroblasts. Molecules of 32 and 34 kilodaltons, which are identified by this antibody have been isolated from neural tissue. This antibody should be very useful in basic research on malignancies of neural origin and possibly for clinical applications. Dr. Seeger has also produced another monoclonal antibody of interest because it detects a determinant on the human Thy-1 molecule and is found primarily on tumors which arise from normal cells whose lineages can also express Thy-1 (14). In this way it is similar to the previously discussed work of Dr. Pellegrino on Ia antigens.

Human ovarian cancer has been a focus of the laboratory of Dr. Lloyd. He has developed a number of monoclonal antibodies utilizing ovarian cancer cell lines as the source of antigen. Most reagents showed broad specificity for carcinomas, and one specifically reacted with ovarian tumors (4/8) and a uterine tumor (1/1). These monoclonals may serve to identify carcinoma-associated antigens and would be key to their isolation and characterization. More importantly, Dr. Lloyd has purified a pregnancy-associated glycoprotein (OvC-1). Two monoclonal antibodies have been generated which specifically detect this 50 kilodalton glycoprotein, which has also been detected on certain tumors (15). A radioimmunoassay is being developed to detect circulating OvC in patients' sera.

During this past year, a second workshop on monoclonal antibodies to human melanoma antigens was held under the auspices of the Immunology Program.



Ten participating laboratories agreed to exchange independently-produced antibodies to melanoma tumor cells. Each laboratory then tested these reagents for their tumor cell and normal tissue specificities, their functional reactivity and also defined the antigens to which the monoclonal antibodies reacted. At this workshop, the participating laboratories discussed their findings: It was agreed that of the 32 monoclonal antibodies studied, 24 identified eleven different antigens on human melanoma cells. These antigens, although not entirely specific to melanoma tumor cells, were found on most melanoma tumors and rarely on non-melanoma tumor cells. Some reacted with a few normal tissues but to a lesser extent. This first attempt to obtain a consensus on human tumor-associated antigens was an important step in human tumor immunology. The participants agreed to continue developing new monoclonal antibodies for future exchanges.

Monoclonal antibodies are proving to be very effective tools for analyzing the various subsets of cells of the immune system. It has been demonstrated that cytotoxic T cells are generated when normal human peripheral blood lymphocytes are incubated with autologous EBV-infected B cells in vitro (16). This cytotoxic response is restricted by the major histocompatibility complex, and is mediated by a lymphocyte-bearing antigens reactive with monoclonal antibodies OKT3, OKT8, OKT11 and SCL. Monoclonal antibody OKT3, in the absence of complement and in low concentration, blocks the specific cytotoxic function of these cells against autologous EBV-infected targets. In contrast, monoclonal antibodies OKT8, OKT11 and SCL had marginal or no effect in blocking cytotoxic function (17). All of these monoclonal antibodies abolished cytotoxic cell function in the presence of complement. The ability of OKT3 to block killer cell function appears to reside in its ability to recognize a particular molecule on the cytotoxic cell which could be involved in antigen recognition or effector cell function.

Monoclonal antibodies are very useful for the identification and separation of cell subsets which perform distinct immunologic functions. Monoclonal antibody plus complement-mediated lysis has been used to delete populations of cells when the appropriate monoclonal antibodies are cytotoxic. However, cells bearing the antigenic markers are not always killed efficiently and not all immunoglobulin classes are cytotoxic. Monoclonal antibodies can be used to enrich for cell populations by "panning" on antibody-coated dishes (18). A modification of this technique involves positive selection of lymphocytes labeled by immunofluorescent staining with monoclonal antibodies, followed by fractionation of fluorescence-positive cells on plates coated with antiluorescein antibody. This procedure facilitates isolation of highly viable and functional human T lymphocyte subsets capable of mediating help and suppression of immunoglobulin synthesis in vitro (19).

A significant problem in understanding in vivo regulation of the T cell response to tumors is the heterogeneity of the response elicited. More than one phenotype and functional subclass of lymphoid cells is evoked in response to tumor and other membrane antigens (20). Dr. Mulé, working with Drs. K.E. and I. Hellstrom, has demonstrated that when small tumor pieces were implanted into tumor-immune mice, the long-lived small lymphocytes that selectively localized into those tumors were predominantly Thy-1<sup>+</sup>, Lyt 1<sup>+</sup>2<sup>-</sup> (21). It is still unclear, however, what the role of this cell subset is in tumor rejection. These cells

may not be directly cytotoxic but may be immunoregulatory cells that are important in a suppressor cell pathway. Tumor-elicited T cells which bear the Lyt-1<sup>+</sup>2<sup>+</sup> phenotype may show direct cytotoxicity *in vitro* (22). The development of cloned T cell lines of defined phenotype, which can be determined with a panel of monoclonal antibodies, will permit more detailed studies of effector mechanisms by which the growth of a tumor is controlled.

Dr. Gershon and co-workers have recently reported the existence of a contra-suppressor effector cell which, although it expresses the Ly-1<sup>+</sup>2<sup>-</sup> phenotype as do helper cells, can easily be distinguished from them by various criteria (23-25). When contrasuppressor effector cells are added to the suppressor cell population, suppression of the helper cells, is markedly reduced. This is not due to inhibition of the suppressor cells because when the helper cells are taken from the chamber after they have sojourned with the cellfree products of suppressor and contrasuppressor cells, they are now resistant to fresh suppressor cells. The contrasuppressor cell expresses an I-J determinant which has not been found on helper cells.

The discovery that interleukin 2 (IL-2 or T cell growth factor) can control T cell proliferation has greatly facilitated the development of cloned continuous T cell lines. Immunochemical studies of this factor have been greatly aided by isolation and characterization of monoclonal antibodies reactive against a determinant shared by murine and human IL-2 (26). Recent studies have demonstrated that immature thymocytes, long thought to be immunoincompetent, will respond to mitogenic/antigenic stimuli in the presence of IL-2. However, if the stimulus itself is deficient in triggering helper cell responses, a separate differentiating/maturation factor is required in order for immature cells to mature to the point where they are capable of responding to the proliferation inducing effects of IL-2 (27). Monoclonal antibodies to IL-2 have been instrumental in serologically defining the role that IL-2 plays in both the differentiation of cytolytic effector cells and the augmentation of natural killer cell activity (28).

Monoclonal antibodies have been used to study the differentiation of lymphoid progenitor cells. Drs. Kersey and LeBien have developed several monoclonal antibodies which are being used to study lymphoid cell differentiation and function. A monoclonal antibody produced by immunization against HSB-2 T cell leukemia has been found to bind to greater than 90% of E-rosette positive lymphocytes. This antibody, designated TA-1, has been found to bind to NK cells and monocytes as well as to T lymphocytes and binds to a 170/110 kilodalton bimolecular complex. TA-1 has been chemically linked to intact ricin molecules; this conjugate can inhibit specific T cell functions, including mixed lymphocyte reaction and mitogen induced proliferation (29). Drs. Kersey and LeBien have made substantial progress toward characterization of cells of early B cell lineage using monoclonal antibody analysis. Monoclonal antibody BA-1 has been produced by immunization with NALM-6 M-1 acute lymphoblastic leukemia cell line. This antibody does not bind T leukemias and is therefore useful for discriminating T versus non-T leukemias. Of a total of 51 cases of ALL studied, 41 were found to bind this antibody. Preliminary analysis of the leukemias binding the BA-1 antibody indicates that they have a significantly better prognosis than those which do not. Immunochemical studies indicate that the BA-1 antibody immunoprecipitates a protein of 30 kilodaltons, is an IgM and fixes complement (30). Several

other monoclonal antibodies have been produced and are also being studied for their usefulness in diagnosis/prognosis/treatment of acute lymphoblastic leukemia.

Dr. Cooper and his colleagues also have been using monoclonal antibodies to study differentiation of immune cells and to analyze their regulatory interactions. Studies employing monoclonal antibodies to antigens on T cells or macrophages revealed that most of the Fc (IgG) receptor-positive E-rosetting ( $T_H$ ) cells in humans do not express T cell antigens but instead share an antigen with monocytes, indicating that this subpopulation of cells may represent human natural killer (NK) cells. They have recently developed a monoclonal antibody (HNK-1) to an antigen on human NK and K cells and have shown that most  $T_H$  cells express this antigen, as do many cells in the "null" blood mononuclear subpopulation (31). Circulating HNK-1<sup>+</sup> cells increase in frequency as a function of age, being less than 1% of blood mononuclear cells in newborns and about 15% in young adults. HNK-1<sup>+</sup> cells are present in bone marrow, migrate selectively to spleen, and comprise less than 1% of thymocytes and lymph node cells. Morphologically, HNK-1<sup>+</sup> cells were defined as homogeneous populations of lymphocytes with abundant cytoplasm containing azurophilic granules, features that were previously described as characteristic of most  $T_H$  cells.

Studies of cell surface markers on natural killer cells have lagged behind those studies on T and B cells because of the difficulty in producing monoclonal antibodies specific for these cell types. Some studies still rely on the use of polyvalent heterologous antisera, while others are using combinations of antiserum and monoclonal antibodies to characterize these naturally occurring cells. The original finding that natural cell-mediated cytotoxicity in mice is mediated by at least two distinct types of effector cells, has been further confirmed. The original distinction between natural killer (NK) and natural cytotoxic (NC) cell populations was based on the observation that NC cells lysed solid tumors and required 12 to 24 hours for lysis, while NK cells lysed lymphoid tumors and required only 4 hours for lysis. The heterogeneity of effector populations involved in natural cell-mediated cytotoxicity is further substantiated by Dr. Stutman and colleagues, who determined that antisera directed to antigens present on NK cells such as Qa-2,3, Qa-5 (available as monoclonal antibody), NK-1.2, Ly-11 and Asialo GM 1, fail to eliminate NC cell activity from murine spleen cells (32, 33). These studies, and others reported previously, indicate that it is difficult to ascribe NK or NC cells as belonging to a defined cell lineage and that natural cell-mediated cytotoxicity is not the function of a single cell type but is a heterogeneous population of distinct cell types.

### Immunologic Intervention

Most of the research involving monoclonal antibodies specifically for diagnostic or therapeutic applications is supported in the Diagnosis or Biological Response Modifiers Programs, respectively. However, basic studies of immunologic mechanisms have often led to findings which have obvious therapeutic or diagnostic implications. For example, Dr. Levy and co-workers have been developing monoclonal antibodies against leukemia and lymphoma cells and have been using a panel of antibodies to phenotype leukemias and lymphomas and to explore models of therapy. For each of the interesting monoclonal antibodies they develop, a detailed



study is made of the cell surface molecule recognized by the antibody. One such antibody, L-17F12, detects an antigen present on 95-100% of human peripheral T lymphocytes, the majority of thymocytes and acute lymphocytic leukemia T cells but not on B cells, B cell lines or monocytes. The antigen recognized by this antibody is a cell surface glycoprotein of 67 kilodaltons (34). Expression of this antigen on normal T cells was not diminished by prolonged exposure in vitro to various T cell stimuli. L17F12 is cytotoxic for T cells in the presence of complement, providing the basis for depletion of T cells from heterogeneous populations. The data suggest that this monoclonal antibody recognizes a specific T cell differentiation protein. Increased amounts of the antigen have been found on the malignant cells of patients with cutaneous T cell lymphomas and some T cell leukemias. These basic studies have led to the development of a protocol using this monoclonal antibody for the treatment of T cell neoplasms (35). Immunologic monitoring of one patient indicated that free antigen became detectable during treatment, with maximum amounts found 24 hours after the beginning of treatment. Antigenic modulation was observed, and was found to be both time and antibody-dose dependent. At the time maximum free antigen was found, however, no antigenic modulation was observed on circulating cells. This does not exclude the possibility that cells sequestered in extravascular compartments could have modulated and shed antigen. In this patient, a weak and clinically insignificant host anti-mouse antibody response was found five days after the first treatment.

To increase the cytotoxicity of monoclonal antibodies to the target cell, many investigators are studying methods to chemically link drugs, toxins, or radio-nuclides to monoclonal antibodies. Dr. Houston has synthesized and characterized conjugates of monoclonal antibody Thy-1.1 and ricin A chain (36). Ricin A chain acts to inhibit protein synthesis only if it penetrates the plasma membrane, and this requires the participation of the B chain. The two chains are held together in ricin by a single disulfide bond. Dr. Houston has shown that the addition of A chain to mouse leukemia cells, after the cells were first reacted with the purified ricin B chain, also results in efficient inhibition of protein synthesis. Dr. Vitetta and colleagues have demonstrated the effectiveness and specificity of an anti-immunoglobulin-ricin A chain conjugate in eradicating BCL<sub>1</sub> tumor cells from infiltrated murine bone marrow (37). The murine BCL<sub>1</sub> tumor is in many respects similar to the prolymphocytic variant of human chronic lymphocytic leukemia. The malignant cells are of B cell origin and carry immunoglobulin on their surface. In these studies, an affinity-purified polyvalent rabbit anti-mouse immunoglobulin, coupled to the A chain of ricin, was used to selectively kill tumor cells in spleen cell populations from BCL<sub>1</sub> bearing mice. The remaining cells were tested for residual tumor cells by adoptive transfer into normal recipient mice. Since injection of only 10 BCL<sub>1</sub> cells into normal mice will induce tumor formation, the adoptive transfer assay represents a very sensitive measurement of the number of residual viable tumor cells in a treated cell population. Similarly, treatment of bone marrow cells from BCL<sub>1</sub>-bearing mice with antibody-A chain conjugates eliminated tumor cells but not stem cells from the marrow. This procedure was much more effective for removal of tumor cells than was the use of antiserum plus complement, which required 250 times more antibody for optimal cytotoxicity. This treatment has promise for the eradication of residual tumor cells in autologous bone marrow before it is transplanted back to the leukemia patient to reconstitute the hematopoietic system. Although these studies used polyvalent rabbit antiserum, they are noteworthy because they indicate that innovative research is being done even

when the appropriate monoclonal antibodies may not yet be available. Ongoing studies applying monoclonal antibody technology to these procedures should result in even greater specificity of response.

The development of monoclonal antibodies which can detect minimal numbers of tumor cells in clinical specimens has great implications for immunodiagnosis. Drs. Reynolds and Smith (38) have developed a monoclonal antibody, HSAN 1.2, which has a high degree of specificity for human neuroblastoma cells and does not react with normal blood or bone marrow, fibroblasts, leukemias and lymphomas, rhabdomyosarcomas, oat cell carcinomas, glioma or melanomas. Weak binding was detected to adult and fetal brain; other normal tissues did not bind the antibody. Specificity of binding was determined by either radioimmunoassay on fresh or glutaraldehyde-fixed cell suspensions or by immunofluorescence assay on viable cell suspensions. The lack of binding to hematopoietic cells permits the development of a sensitive assay for the detection of neuroblastoma cells in bone marrow. Surveys of marrow aspirates from patients with neuroblastoma have detected tumor cells when the sample appears to be normal by light microscopic examination. The same procedure has been applied to blood samples with similar results. This method may be useful for the detection of sub-clinical metastases and for monitoring patients for recurrent disease during and after therapy. The ability of this antibody to discriminate neuroblastoma from hematopoietic cells may also be useful for removing neuroblastoma cells from bone marrow prior to using it for autologous bone marrow transplantation.

Although most monoclonal antibodies are not directly cytotoxic to tumor cells in the presence of complement, Dr. Billing and co-workers have reported the development of a monoclonal antibody cytotoxic to a human leiomyosarcoma (39). They have developed another monoclonal antibody, CALL2, which is highly cytotoxic to T ALL cells but does not react with normal peripheral-blood T and B lymphocytes, monocytes, granulocytes, spleen cells, thymocytes, platelets and other acute and chronic leukemia cells (40). The CALL2 antibody does not react with bone marrow stem cells of the myeloid series. Both of these antibodies appear to have sufficient specificity to be useful in passive immunotherapy trials.

#### Meeting Support

The following meetings, conferences and workshops received support from the Immunology Program in FY 1982:

"Fifth Ir Gene Workshop" - Bethesda, Maryland  
September 1981

"Conference on IgD Structure and Function" - New York, New York  
January 1982

"IX International Reticuloendothelial Society Congress" - Davos, Switzerland  
February 1982

"Conference on B and T Cell Tumors" - Keystone, Colorado  
April 1982



"Workshop on Macrophage Activation" - Hilton Head, South Carolina  
May 1982

"Conference on Mononuclear Cell and Antibody Networks" - Saxtons River, Vermont  
July 1982

"Third International Lymphokine Workshop" - Philadelphia, Pennsylvania  
August 1982

"Thirteenth International Cancer Congress" - Seattle, Washington  
September 1982

"Immune Network" - New York, New York  
September/October 1982

"Fifteenth International Leucocyte Culture Conference" - Pacific Grove, Calif.  
December 1982

"Workshop on Rabbit Immunogenetics and Immunobiology" - Memphis, Tennessee  
April 1983

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FISCAL YEAR 1982

IMMUNOLOGY PROGRAM

SUMMARY OF GRANTS BY SUBCATEGORY

(Includes P01, R01, R23, Grants)

Dollars in Thousands

Subcategory	No. of Grants	Total Costs Awarded
Myeloma Proteins	22	\$ 2,566
Cell Surface Antigens	73	9,046
Cell Surface Determinants of		
Lymphocytes & Macrophages	46	5,069
Humoral Factors Other Than Antibody	40	3,770
Tumor-Related Antibodies	15	1,441
Immunobiology of Sarcomas,		
Carcinomas & Melanomas	9	848
Host/Tumor Immunopathology	13	1,375
Effects of Disease on Immune		
Function	37	3,514
Immunotherapy: Mechanisms Rather		
Than Therapeutic Result	13	1,705
Lymphocytes	97	14,208
Monocytes & Macrophages	31	3,635
Malignancies of the Immune System		
(Lymphoma/Leukemia)	31	3,178
Immune Surveillance	28	2,640
Immunotherapy in Animal Models	9	750
Bone Marrow Transplantation	3	388
	467	\$54,133

# MYELOMA PROTEINS

R01-CA-04946 Bosma	Hidden Immunoglobulin Allotypes in Mice Institute for Cancer Research
R01-CA-08497 Putnam	Abnormal Proteins in Multiple Myeloma Indiana University, Bloomington
R01-CA-10056 Solomon	Proteins in Multiple Myeloma & Related Blood Diseases University of Tennessee, Knoxville
R01-CA-12421 Adams	Structure of Immunoglobulin Messenger RNAs and Genes Walter and Eliza Hall Inst. Medical Research
R01-CA-13014 Beychok	Proteins of Plasma Cell Cancers Columbia University
R01-CA-16858 Morrison	Genetics and Biochemistry of Myeloma Ig Production Columbia University
R01-CA-19616 Edmundson	Immunoglobulins in Multiple Myeloma and Amyloidosis University of Utah
R01-CA-20088 Witz	Functions of Tumor-Bound Ig and Ig-Receptor Molecules Tel Aviv University
R01-CA-22105 Tomasi	Murine Immunoglobulins and B Cell Differentiation University of New Mexico, Albuquerque
R01-CA-24432 Haber	Sequence, Shape and Specificity of Antibodies Massachusetts General Hospital
R01-CA-25048 Sarma	X-Ray Crystal Structure and Function of Proteins State University New York, Stony Brook
P01-CA-25319 Beychok	Synthesis of Human Myeloma Variable Domains in E. coli Columbia University
R01-CA-25507 Gearhart	B Cell Expression of Immunoglobulin V and C Regions Carnegie Institution of Washington, D.C.
R01-CA-25754 Storb	Control of Immunoglobulin Synthesis University of Washington
R01-CA-28871 Green	Ig Processing by Lymphocyte Endoplasmic Reticulum St. Louis University
R01-CA-29634 Kemp	Immunoglobulin mRNA and Genes in T Cell Tumors Walter and Eliza Hall Inst. Medical Research
R01-CA-29679 Sibley	Genetic Analysis of Membrane Immunoglobulin University of Washington

R01-CA-31013 Blattner	Immunoglobulin Genes of Normal and Leukemic Human DNA University of Wisconsin, Madison
R01-CA-31683 Milcarek	Immunoglobulin Gene Expression in Myeloma Mutants Columbia University
R01-CA-32044 Robinson	Human Monoclonal Antibodies from EBV Transformed Cells Yale University
R01-CA-32497 Cannon	Structure and Genetics of Antibody Variable Regions University of Massachusetts Medical School
R01-CA-32582 Lamm	Studies on Secretory Immunoglobulin Case Western Reserve University

#### CELL SURFACE ANTIGENS

R01-CA-12851 Sanders	Embryonic & Virally Induced Tumor-Cell Membrane Antigens University of Texas, Austin
R01-CA-13070 Dawson	Immunity to Human Cancer--Functional Components Duke University
R01-CA-13287 Hyman	Genetic Basis of Antigenic Variation Salk Institute for Biological Studies
R01-CA-13844 Bystryn	Isolation of Tumor Antigens of Human Melanoma New York University
R01-CA-14054 Klein	Malignant Behavior and Cellular Antigen Expression Caroline Institute
R01-CA-18470 Knowles	Antigenicity and Tumorigenicity of Somatic Cell Hybrids Wistar Institute of Anatomy and Biology
R01-CA-18600 Coddington	Masking of Antigens at Cancer Cell Surfaces Massachusetts General Hospital
R01-CA-18609 Acton	Biological Role of Alloantigens University of Alabama in Birmingham
R01-CA-19149 Hellstrom	Transplantation Antigenicity of Virus Induced Tumors Fred Hutchinson Cancer Research Center
R01-CA-19224 Hakomori	Relation of Blood Group and Human Tumor Antigen Fred Hutchinson Cancer Research Center
P01-CA-19765 Old	Human Cancer Serology Sloan Kettering Institute for Cancer Research
R01-CA-20168 Little	Neoplastic and Normal Cell Thymus-Leukemia Antigens Jewish Hospital of St. Louis

R01-CA-21223 Levy	Antitumor Antibodies Generated In Vitro Stanford University
R01-CA-21445 Lloyd	Antigens of Malignant Melanoma and Other Human Tumors Sloan Kettering Institute for Cancer Research
P01-CA-22507 Dupont	Immunogenetics of the Major Histocompatibility Complex Sloan Kettering Institute for Cancer Research
R01-CA-22540 Springer	Nature of T-Specific Human Carcinoma Antigens Evanston Hospital
R01-CA-22674 Coggin	Characterization of Fetal Antigens in Tumors University of South Alabama
R01-CA-22794 Seeger	Human Neuroblastoma Cell Surface Antigens University of California, Los Angeles
P01-CA-23115 Frenkel	New Immunologic Approaches to Lymphoid Neoplasms University of Texas Health Science Center, Dallas
R01-CA-23404 Hirshaut	Serologic Screening for Contagion of Human Sarcomas Sloan Kettering Institute for Cancer Research
R01-CA-23568 Croce	Immunoresponse to Human Surface Antigens Wistar Institute of Anatomy and Biology
R01-CA-23770 Haughton	Antigen Induced Lymphoma of Mice University of North Carolina, Chapel Hill
R01-CA-24024 Tom	In Vitro Generation of Immunity to Human Colon Cancer University of Texas Health Science Ctr., Houston
R01-CA-24263 Kennett	Hybridomas: Production and Genetic Applications University of Pennsylvania
R01-CA-24358 Billing	Leukemia Associated Antigens University of California, Los Angeles
R01-CA-25134 Poretz	Lymphocyte Surface Glycoconjugates Rutgers the State University, New Brunswick
R01-CA-25139 Warren	Study of Group 5 Antigens in Hematologic Malignancies Fred Hutchinson Cancer Research Center
R01-CA-25154 Chauvenet	Tumor-Specific Immunity and Histocompatibility Complex University of Texas Hlth. Sci. Ctr., San Antonio
R01-CA-25171 Callahan	Cell Surface Antigens of Murine Tumors Scripps Clinic and Research Foundation
R01-CA-25558 Hellstrom	Cell Surface Antigens of Chemically Induced Sarcomas Fred Hutchinson Cancer Research Center

P01-CA-25874 Koprowski	Human Melanoma and Tumor Specific Monoclonal Antibodies Wistar Institute of Anatomy and Biology
R01-CA-26184 Lloyd	Antigens of Human Ovarian Tumors Sloan Kettering Institute for Cancer Research
R01-CA-26321 Allison	Cell-Surface Antigens of Murine Tumors University of Texas System Cancer Center
R01-CA-26479 Fuji	Immune Functions of Tumor Cell Variants Roswell Park Memorial Institute
R01-CA-26891 Allison	Surface Antigens of Rat Hepatocellular Carcinomas University of Texas System Cancer Center
R01-CA-27124 Kahan	Molecular Approaches to Human Colon Cancer University of Texas Health Science Ctr., Houston
R01-CA-27471 Shinitzky	Modulation of Cellular Responses by Membrane Fluidity Weizmann Institute of Science
R01-CA-27534 Busch	Nucleolar Antigens of Human Cancer Cells Baylor College of Medicine
R01-CA-27628 Milgrom	Tumor-Specific and Tumor-Associated Antigens State University of New York at Buffalo
R01-CA-27841 Brown	Antigens of Chemically Transformed Mouse Fibroblasts Fred Hutchinson Cancer Research Center
P01-CA-28166 Edgington	Molecular Immunology and Pathobiology of Neoplasia Scripps Clinic and Research Foundation
R01-CA-28212 Minden	Antisera for Human Tumor-Associated Antigens National Jewish Hospital & Research Center
R01-CA-28230 Hook	Swine Melanoma Antigens: Isolation and Evaluation University of Missouri, Columbia
R01-CA-28420 Reisfeld	Molecular Profile of Human Melanoma Antigens Scripps Clinic and Research Foundation
R01-CA-28448 Levine	Forssman Antigen/Antibody and Human Adenocarcinoma Sloan Kettering Institute for Cancer Research
R01-CA-28461 De Leo	Cell Surface Antigens of Mouse Sarcomas Sloan Kettering Institute for Cancer Research
R01-CA-28564 Carey	Human Squamous Cell Carcinoma: Culture and Serology University of Michigan
R01-CA-28619 Hirshaut	Purification of Human Sarcoma Heterophile Antigens Sloan Kettering Institute for Cancer Research



R01-CA-28732 Williams	Studies of Immune Complexes in Patients with Leukemia University of New Mexico, Albuquerque
R23-CA-29377 Tsang	Tumor Associated Antigens of Human Osteosarcoma Medical University of South Carolina
R01-CA-29516 Bernstein	Human Antibodies to Melanoma Pacific Northwest Research Foundation
R23-CA-29539 Young	Role of Glycolipids in Immune Cell Function University of Virginia
R01-CA-29863 Michaelson	Immunochemical Genetics of Murine Alloantigens New York University
R01-CA-29886 De Witt	Tumor-Host Associated Immunological Specificities University of Utah
R01-CA-29909 Dreyer	Molecular Characterization of Human Tumor Markers California Institute of Technology
R01-CA-29989 Pollack	HLA Alloantigens on Cultured Human Tumor Cell Lines Sloan Kettering Institute for Cancer Research
R01-CA-30070 Anderson	Carcinoma Associated Antigens and Immunoglobulins Northwestern University
R01-CA-30209 Alpert	Immunochemical Studies of Gastrointestinal Cancer Baylor College of Medicine
R01-CA-30266 Gooding	Membrane Antigen Organization in Tumor Immunity Emory University
R01-CA-30501 Mills	Isolation and Characterization of the Common ALL Antigen City of Hope National Medical Center
R01-CA-30561 Spellman	Tumor Associated Antigens of UV-Induced Tumors University of New Mexico, Albuquerque
R01-CA-31378 Fishman	Immunochemical Studies of Placental Alkaline Phosphatase La Jolla Cancer Research Foundation
R01-CA-31620 Bonavida	Inappropriate H-2 (K/D) and IE/C Antigens on Tumors University of California, Los Angeles
R01-CA-31740 Gold	Organ Specific Antigens of Gastrointestinal Tissues University of Kentucky
R01-CA-31828 Ricardo	Immune Response to Syngeneic Leukemic B Cell Antigens University of Tenn. Center Health Science
R01-CA-32132 Anderson	Cross-Reacting Antigens on Spermatozoa and Cancer Cells Sidney Farber Cancer Institute

R01-CA-32578 Garver	Characterization of Tumor Antigen on Leukemia Cells Medical College of Georgia
R01-CA-32609 Ferrone	Immunochemical Characterization of Antigens in Melanoma Columbia University
R01-CA-32632 Klock	Complex Carbohydrate Chemistry in Leukocytes Institutes of Medical Sciences
R01-CA-32635 Pellegrino	Antigenic Profile of Human Leukemic Cells Columbia University
R01-CA-32896 Pesando	Human Leukemia-Associated Antigens Sidney Farber Cancer Institute
R01-CA-32925 Quaranta	Pancreatic Tumor Antigens Defined by Monoclonal Antibody Scripps Clinic and Research Foundation
R01-CA-33014 Eskinazi	Oral Immunopathology: Oral Squamous Cell Carcinoma University of California, Los Angeles

#### CELL SURFACE DETERMINANTS OF LYMPHOCYTES AND MACROPHAGES

R01-CA-04681 Herzenberg	Genetic Studies with Mammalian Cells Stanford University
R01-CA-14061 Gilmour	Chicken Lymphocyte Alloantigens and Viral Oncogenesis New York University
R01-CA-15146 Gasser	Genetic Control of the Immune Response University of Pennsylvania
R01-CA-17680 Collins	Genetics, the Lymphocyte and Tumor Regression University of New Hampshire
R01-CA-18640 Silvers	Behavior of Weak Transplantation Antigens University of Pennsylvania
R01-CA-18659 Gill	Chemical Genetic and Cellular Aspects of Immunogenicity University of Pittsburgh
R01-CA-18734 Jones	Immunologic Studies Related to Malignancy University of Colorado Health Sciences Ctr.
R01-CA-20473 Boyse	Immunogenetics of the TLA Region of Chromosome 17 Sloan Kettering Institute for Cancer Research
R01-CA-20500 Cullen	Structural and Serological Studies on Ia Antigens Washington University
R01-CA-20820 Freed	Structural Studies of the Products of the H-2 Complex Johns Hopkins University

P01-CA-21112 Osserman	Clinical and Basic Studies of Plasma Cell Dyscrasias Columbia University
R01-CA-21651 Artzt	Teratocarcinoma and Embryonal Tumors: Surface Antigens Sloan Kettering Institute for Cancer Research
R01-CA-22131 Boyse	Immunogenetics of Ly Systems Sloan Kettering Institute for Cancer Research
R01-CA-22662 Frelinger	Genetics and Function of Murine Ia Antigens University of Southern California
R01-CA-23027 Flaherty	Immunogenetic Mapping of the Cell Surface New York State Department of Health
R01-CA-23469 Yang	Cells Involved in Spontaneous Regression of Tumors University of Connecticut, Storrs
R01-CA-24067 Anderson	Fc Receptor Structure and Function University of Rochester
R01-CA-24433 Sears	Structures of Histocompatibility-2 Membrane Antigens University of California, Santa Barbara
R01-CA-24437 Esselman	Expression of T Lymphocyte Differentiation Antigens Michigan State University
R01-CA-24473 David	Genetics & Functions of (H-2 Linked) I Region Mayo Foundation
R01-CA-25038 Cramer	Major Histocompatibility Complex in the Wild Rat University of Pittsburgh
R01-CA-25044 Hickman	Surface IgM of Malignant Lymphocytes and Plasma Cells Jewish Hospital of St. Louis
R01-CA-25056 Misnell	Immunoregulatory Effects of Bacterial Substances University of California, Berkeley
R01-CA-25532 Schwartz	Glycolipids of Normal and Transformed Mouse Lymphocytes Eunice Kennedy Shriver Ctr. Mtl. Retardation
R01-CA-25893 Hyman	Cell Surface Molecules: Hematopoietic Differentiation Salk Institute for Biological Studies
R01-CA-26297 McKean	Primary Structure of MHC I Region Associated Antigens Mayo Foundation
R01-CA-27824 Whisnant	Plasma Membrane Composition and Immune Function Duke University
R01-CA-27955 Williams	Role of the H-2NB Gene in Hybrid Resistance to P815 Northwestern University

R01-CA-28992 Decker	Membrane Lectins on Normal and Neoplastic Lymphocytes Medical University of South Carolina
R01-CA-29111 Beisel	Expression of H-2 Antigens on SJL/J Tumors Wayne State University
R01-CA-29194 Rajan	Somatic Cell Genetics of Cell Surface Antigens Yeshiva University
R01-CA-29548 Hansen	Differentiation Antigens on Human Lymphocytes Pacific Northwest Research Foundation
R01-CA-29657 Haran-Ghera	Genetic Control in Leukemogenesis Weizmann Institute of Science
R23-CA-29738 Mitchell	Macrophage Membrane and Immunomodulators University of Southern California
R01-CA-29979 Yamazaki	Immunogenetics of Self-Identification Monell Chemical Senses Center
R01-CA-30654 Morgan	Regulation of Immune Responses by Fc Portion of Antibody Scripps Clinic and Research Foundation
R01-CA-31555 Silver	Structural Studies of Ia Alloantigens Michigan State University
R01-CA-31638 McKean	Characterization of MHC Restricted Antigen Presentation Mayo Foundation
R01-CA-31798 Springer	Murine T Lymphocyte Cell Surface Antigens Sidney Farber Cancer Institute
R01-CA-31799 Springer	Chemistry of Tumoricidal Macrophage Surface Antigens Sidney Farber Cancer Institute
R01-CA-32043 Callahan	T-Cell Recognition of Mutant and Tumor Cell MHC Antigens Scripps Clinic and Research Foundation
R23-CA-32091 Heagy	Monoclonal Antibody Analysis of Cloned/Mutant Killers University of Massachusetts, Amherst
R01-CA-32634 Pellegrino	Urine as Source of Human Cell Surface Markers Columbia University
R01-CA-33555 Todd	Cell Surface Antigens on Human Macrophages Sidney Farber Cancer Institute
R01-CA-34108 Kimura	Structures Related to Function on Cytotoxic T Cells University of Florida
R01-CA-34110 Twomey	Functions of Clonally Derived Human Monocytes Baylor College of Medicine

# HUMORAL FACTORS OTHER THAN ANTIBODY

R01-CA-01786 Deutsch	Human Blood and Tissue Proteins University of Wisconsin, Madison
R01-CA-07191 Rosenau	Nuclear Envelope-Centromere Interactions University of California, San Francisco
R01-CA-15129 Oh	A Serum Immunosuppressive Factor in Cancer Boston University
R01-CA-15585 Zolla-Pazner	A Soluble Mediator of Tumor-Induced Immunosuppression New York University
R01-CA-17643 Smith	Regulation of T-Cell Proliferation and Differentiation Dartmouth College
R01-CA-19148 Hellstrom	Lymphocyte Allogeneic Inhibition and Tumor Immunity Fred Hutchinson Cancer Research Center
R01-CA-19529 Valentine	Cell-Mediated Immunity in Humans: Mechanisms and Uses New York University
R01-CA-22720 Long	H-2 Complex and Susceptibility to Mammary Tumor Virus Hahnemann Med. College & Hospital of Philadelphia
R01-CA-24441 Mayer	Biochemical Studies of Lymphokines and Related Agents Johns Hopkins University
R01-CA-24474 Finke	Killer Assisting Factor in Cell Mediated Lympholysis Cleveland Clinic Foundation
R01-CA-24476 Incefy	T-Lymphocyte Differentiation Sloan Kettering Institute for Cancer Research
R01-CA-24916 Sundharadas	Studies of a Tumor Factor That Affects Macrophages University of Wisconsin, Madison
R01-CA-24974 Goldstein	Chemical & Immunological Characteristics of Thymosin George Washington University
R01-CA-25035 Kamin	Immune Interferons: Production and Role in Immunity University of California, San Francisco
R01-CA-25750 Miller	Structure-Function Relations of Immunoregulatory Protein University of Chicago
R01-CA-25943 Elgert	Immunobiochemistry of Macrophage-Derived Factors Virginia Polytechnic Inst. and St. Univ.
R01-CA-26019 Godfrey	Isolation of Macrophage Agglutination Factor State University New York, Stony Brook



R01-CA-26143 Lint	Control of Complement-Mediated Tumor Cell Cytolysis Rush-Presbyterian-St. Lukes Medical Center
R01-CA-26462 Reiss	Role of Macrophage Arginase in Tumor Immunity University of Colorado Health Sciences Center
R01-CA-26504 Stanley	Regulation of Granulocyte and Macrophage Production Yeshiva University
R01-CA-27629 Paque	"Tumor Immune" RNA: A Biochemical Characterization University of Texas Hlth. Sci. Ctr., San Antonio
R01-CA-27701 Ladisch	Human Immunoregulatory Gangliosides University of California, Los Angeles
R01-CA-27903 Epstein	Interferon as a Mediator of Cellular Immunity University of California, San Francisco
R01-CA-28123 Huang	The Induction of Human Interferon in C-10 Cells Johns Hopkins University
R01-CA-28419 Gillis	Control of Normal and Leukemic T-Cell Proliferation Fred Hutchinson Cancer Research Center
R01-CA-28471 Dvorak	Biology of Solid Tumor Growth and Immune Rejection Beth Israel Hospital
R01-CA-30015 Mortensen	C-Reactive Protein Regulation of Tumor Immunity Ohio State University
R01-CA-30515 Sidell	Immunological Aspects of Retinoids in Human Cancer University of California, Los Angeles
R01-CA-30651 Edwards	Monocyte Tissue Factor: In Vivo and In Vitro Modulation University of Connecticut Health Center
R23-CA-30669 Yung	Growth and Differentiation of Mast Cells and T Cells Sloan Kettering Institute for Cancer Research
R23-CA-30894 Mier	Binding Studies with Purified Human T-Cell Growth Factor New England Medical Center Hospital
R23-CA-30988 Mathews	Tumor Specific Helper Factor(s) Loyola University Medical Center
R01-CA-31394 Lotzova	Effect of Interferon Inducers on NK Cell Cytotoxicity University of Texas System Cancer Center
R23-CA-32319 Cohen	Immunologic Control of Tumor Cell Migration University of Connecticut Health Center
R23-CA-33090 Klostergaard	Biochemistry and Biological Role of Lymphotoxins University of California, Irvine

R01-CA-33168 Incefy	Thymic Peptides, Monoclonal Antibodies and Cancer Sloan Kettering Institute for Cancer Research
R01-CA-33557 Plate	Transplantation Antigen Specific Immunosuppression Rush-Presbyterian-St. Lukes Medical Center
R01-CA-34103 Day	Complement and Immune Complexes in Lymphosarcoma Oklahoma Medical Research Foundation
R01-CA-34120 Schreiber	Molecular Regulation of Macrophage Cytocidal Activity Scripps Clinic and Research Foundation
R01-CA-34121 Huang	Effect of Thymosin on Interferon Induction George Washington University

#### TUMOR RELATED ANTIBODIES

R01-CA-15064 Chu	Immunochemical Studies on Carcinogenic Mycotoxins University of Wisconsin, Madison
R01-CA-20045 Phillips-Quagliata	Antibody Mediated Cell-Cell Interactions New York University
R01-CA-20075 Siskind	Antibody Affinity in Immune Response and Tolerance Cornell University Medical Center
R01-CA-23028 Richards	Molecular Studies of the Immune Response California Institute of Technology
R01-CA-23967 Buchsbaum	Radiolabeled Antibody Tumor Localization University of Minnesota of Minneapolis-St. Paul
R01-CA-26882 Klein	Antibody Responses of Tumor Bearers to Their Tumors University of Florida
R01-CA-28149 Vitetta	Immunotherapy of a B Cell Leukemia (ECL1) University of Texas Health Science Ctr., Dallas
R01-CA-29876 Kaplan	Human Hybridoma Antibodies in Neoplastic Disease Stanford University
R01-CA-29889 Houston	Targeting Antibody-Toxin Conjugates to Leukemia Cells University of Kansas, Lawrence
R01-CA-30313 Slavin	New Approaches to the Therapy of a B Cell Leukemia Hadassah University Hospital
R01-CA-30647 Irie	In Vitro Synthesis of Human Antibodies to Oncofetal Ag University of California, Davis
R01-CA-30663 Collier	Antibody-Directed Tumor Specific Chimeric Toxins University of California, Los Angeles

R01-CA-30990 Bell	Monoclonal Antibodies to Human Lung Carcinoma Antigens Washington University
R01-CA-32619 Ferrone	Monoclonal Antibodies to Human Melanoma Antigens Columbia University
R23-CA-33545 Kamoun	Macrophage Differentiation Antigens and Heterogeneity Oregon Health Sciences University

#### IMMUNOBIOLOGY OF SARCOMAS, CARCINOMAS, AND MELANOMAS

R01-CA-14462 Thorbecke	Properties of Lymphoid Tumor Cells In Vivo and In Vitro New York University
R01-CA-19753 Bonavida	Mixed Leukocyte Tumor Reaction in Syngeneic Systems University of California, Los Angeles
R01-CA-20364 Seigler	Immunodiagnosis of Melanoma Duke University
R01-CA-25214 Gusdon	Immunological Studies of Herpes Induced Fibrosarcoma Wake Forest University
R01-CA-28311 Haughton	Immunobiology of Murine Primary Rous Sarcoma University of North Carolina, Chapel Hill
R01-CA-28611 De Wolf	Teratocarcinoma Tumor Associated Fetal Embryonic Antigen Sidney Farber Cancer Institute
R01-CA-29007 Oettgen	Melanoma Surface Antigens and Cytotoxic T Cells Sloan Kettering Institute for Cancer Research
R01-CA-30461 Mukherji	Clonal Analysis of Cellular Immune Response in Melanoma University of Connecticut Health Center
R23-CA-31732 Cook	Tumor Cell Resistance to Destruction by Effector Cells National Jewish Hospital & Research Center

#### HOST-TUMOR IMMUNOPATHOLOGY

P01-CA-16835 Kyle	Monoclonal Gammopathies--Humoral Immune Status Mayo Foundation
R01-CA-16869 Theilen	Oncornea Cell Membrane Antigens & Its Therapeutics University of California, Davis
R01-CA-17800 Winn	Tumor Immunology Massachusetts General Hospital

R01-CA-23679 Eichwald	Cell Mediated Hyperacute Rejection University of Utah
R01-CA-24196 Fortner	Ultraviolet Carcinogenesis and Immunity Kansas State University
R23-CA-27893 Chander	Bence Jones Protein Properties & Tubulotoxicity New York Medical College
R01-CA-28060 Frost	Immunobiology Metastasis University of California, Irvine
R01-CA-28139 Feldman	The Immunobiology of Tumor Metastasis Weizmann Institute of Science
R23-CA-30110 Galli	Vascular Damage in Skin Allograft and Tumor Rejection Beth Israel Hospital
R01-CA-30169 Friedlaender	Bone Allografts for Surgical Oncology Yale University
R01-CA-30196 Purtilo	Immunopathology of X-Linked Lymphoproliferative Syndrome University of Nebraska Medical Center
R01-CA-30565 Newcom	Growth Factor(s) in Nodular Sclerosing Hodgkin's Disease Oregon Health Sciences University
R01-CA-32577 Wheelock	Studies on Tumor Dormancy and Emergence Hahnemann Med. College & Hospital of Philadelphia

#### EFFECTS OF DISEASE ON IMMUNE FUNCTION

R01-CA-10267 Rosse	Immunological Lysis in Neoplastic Disease Duke University
R01-CA-15462 Argyris	Cell Interactions in Tumor Immunity Upstate Medical Center
R01-CA-17818 Stutman	Tumor Immunity and Tumor-Host Interactions Sloan Kettering Institute for Cancer Research
R01-CA-18234 Roszman	Immunobiology of Primary Intracranial Tumors University of Kentucky
P01-CA-19267 Oettgen	Clinical Immunobiology Program Project Memorial Hospital for Cancer-Allied Diseases
R01-CA-23709 Adler	Induction of Tolerance and Suppressor Cells In Vitro St. Jude Children's Research Hospital
R01-CA-24429 Winkelstein	Immunosuppressants and Lymphocyte Function Montefiore Hospital

R01-CA-24873 Bankhurst	Immunosuppression in Cancer Patients University of New Mexico, Albuquerque
R01-CA-25183 Wood	Immunologic Factors in Central Nervous System Tumors University of Kansas Col. Hlth. Sci. & Hosp.
R01-CA-26169 Bose	Immunosuppression During Acute Avian Leukemia University of Texas, Austin
R01-CA-26268 Dube	Anti-II Antibodies and II Antigens in Carcinomas Evanston Hospital
R01-CA-26760 Weston	Monocyte-Lymphocyte Interactions in Mycosis Fungoides University of Colorado Hlth. Sciences Center
R01-CA-26861 Giovannella	Determination of Malignant Potential of Cultured Cells Stehlin Foundation for Cancer Research
R01-CA-27168 Osband	Histamine H2 Receptor T-Suppressor Cells in Cancer University Hospital
R01-CA-27390 Spence	Ethylnitrosourea-Induced Rat Gliomas University of Washington
R01-CA-28167 Ekstedt	Tumor Enhancement in Lectin Treated Mice Northwestern University
R23-CA-28433 Ostenson	Identification of Ag Specific Suppressor Cells in Man Fred Hutchinson Cancer Research Center
R23-CA-29155 Locke	Stress and Human Cell-Mediated Immunity Beth Israel Hospital
R01-CA-29200 Guerry	Autologous Immunity to Human Cultured Melanoma University of Pennsylvania
R01-CA-29752 Devens	Immune Response Modulation by Tumor Promoter University of California, Riverside
R01-CA-29906 Kadish	Mechanisms of Immunoregulation in Human Cancer Yeshiva University
R01-CA-30020 Aisenberg	The Cell Surface Phenotype of Malignant Lymphoma Massachusetts General Hospital
R01-CA-30088 Dray	Synergy of Tumor Chemotherapy and Host Immunity University of Illinois Medical Center
R23-CA-30160 Chi	Cytotoxic and Suppressor Cells in the Chicken East Tennessee State University
R01-CA-30187 Bloom	Regulation of Cell-Mediated Cytotoxicity Mechanisms University of California, Los Angeles



R01-CA-30457 Koros	Immunoregulation of Human Tumor Growth in Nude Mice University of Pittsburgh
R01-CA-30660 Keller	Immunoregulatory Dysfunctions in Non-Hodgkin's Lymphoma Medical College of Wisconsin
R01-CA-30920 Uhr	Immunosuppression in Murine Chronic Lymphocytic Leukemia University of Texas Health Sciences Ctr., Dallas
R01-CA-31226 Meyers	Tolerance and Immunity to Avian RNA Tumor Viruses and VI Mayo Foundation
R01-CA-31336 Stackpole	Antigen Evasion as a Tumor Escape Mechanism New York Medical College
R01-CA-31547 Olsen	Immunosuppressive Properties of Retrovirus Protein Ohio State University
R01-CA-31837 Prehn	Mechanisms of Carcinogenesis Santa Clara Valley Medical Center
R01-CA-32070 Platsoucas	Cell Interactions in Leukemia Sloan Kettering Institute for Cancer Research
R01-CA-32275 Lynch	Immunoregulation of Murine Myeloma University of Iowa
R01-CA-32563 Hoover	Pathogenesis of Preleukemic Aplastic Anemia Colorado State University
R01-CA-32630 Johnson	Relationship of Leukemogenesis and Immune Responsiveness Scripps Clinic and Research Foundation
R01-CA-34098 Veltri	Immunoregulatory Factors in Head and Neck Cancer West Virginia University

#### IMMUNOTHERAPY--MECHANISM RATHER THAN THERAPEUTIC RESULT

R01-CA-11605 Simmons	Immunological Reactivity in Special Circumstances University of Minnesota at Minneapolis
R01-CA-20484 Bortin	Specific Adoptive Immunotherapy of AKR Leukemia Mount Sinai Medical Center
R01-CA-26138 Harris	Immune Testing in Lung Cancer During Immunotherapy Rush University
R01-CA-26738 Zarling	Cellular Immunity to Tumors University of Minnesota of Minneapolis-St. Paul
R01-CA-27625 McCune	Hybrid Tumor Cell Immunotherapy University of Rochester

R01-CA-28441 Terman	Extracorporeal Immunoabsorbents in Immunotherapy Baylor College of Medicine
R01-CA-28941 Deeg	Resistance and Sensitization--Role of Lymphocyte Subsets Fred Hutchinson Cancer Research Center
R01-CA-29039 Raso	Cell Specific Antibody/Ricin A Chain Cytotoxins Sidney Farber Cancer Institute
R01-CA-29328 Parkman	Control of Graft-Versus-Host Disease Sidney Farber Cancer Institute
R01-CA-31787 Thomas	Irradiation and Marrow Transplantation in Large Animals Fred Hutchinson Cancer Research Center
R01-CA-32123 Mastrangelo	Augmentation of Human Immunity by Cyclophosphamide Fox Chase Cancer Center
R01-CA-33060 Jones	Removal and Analysis of Immune Complexes from Tumor Cats Sloan Kettering Institute for Cancer Research
R01-CA-33084 Greenberg	Mechanisms of Murine Tumor Eradication by Immunotherapy University of Washington

#### LYMPHOCYTES

R01-CA-03367 Trentin	Immunogenetic Resistance to Lymphoma-Leukemia Baylor College of Medicine
P01-CA-12800 Fahey	Immune Functions and Cancer University of California, Los Angeles
R01-CA-12844 Cudkowicz	Controls of Proliferation Specific for Leukemias State University of New York at Buffalo
R01-CA-13339 Weksler	Host Defense Against Lymphoblastic Leukemia Cornell University Medical Center
R01-CA-13396 Miller	Immunogenesis from Bone Marrow Cells Michigan State University
R01-CA-14049 Amos	Cell-Mediated Immunity to Ascites Tumors Duke University
R01-CA-14216 Gershon	Characterization of Lymphoid Populations in Cancer Yale University
P01-CA-14723 Benacerraf	Study of Experimental Cancer Immunology Harvard University
P01-CA-15822 Wilson	Immunobiology of Normal and Neoplastic Lymphocytes University of Pennsylvania

P01-CA-16673 Cooper	Cell Differentiation Studies in Cancer Immunobiology University of Alabama in Birmingham
R01-CA-16885 Ruddle	Propagation of Thymus-Derived Lymphocyte Lines Yale University
R01-CA-17034 Gajl-Peczalska	Immunology of Human Lymphoid Tumors of B-Cell Origin University of Minnesota of Minneapolis-St. Paul
P01-CA-17404 Choi	Immunobiology, Immunodeficiency, and Cancer Sloan Kettering Institute for Cancer Research
R01-CA-17531 Manning	Mechanism and Uses of Anti-Ig Immunosuppression University of Wisconsin, Madison
R01-CA-17673 Hoffmann	Regulation of Immunity by Antibody and B-Cells Sloan Kettering Institute for Cancer Research
R01-CA-17733 Trowbridge	Lymphocyte Antigens: Structure, Function and Synthesis Salk Institute for Biological Studies
R01-CA-19170 Bernstein	Mechanisms of BCG-Mediated Suppression of Tumor Growth Fred Hutchinson Cancer Research Center
R01-CA-19334 Dennert	Antigen Receptor of Continuous T Killer Cell Line Salk Institute for Biological Studies
R01-CA-20105 Wohlgenuth	Information from Immunological Reaction Tables University of Maine at Orono
R01-CA-20531 Yunis	Genetic Analysis of Normal and Malignant Lymphocytes Sidney Farber Cancer Institute
R01-CA-20823 Rosse	Lymphocyte Production and Traffic in the Bone Marrow University of Washington
R01-CA-22126 Daynes	Ultraviolet Light Radiation and Immunoregulation University of Utah
R01-CA-22241 Scheid	T Cell Development: Immunogenetics, Defects, Therapy Sloan Kettering Institute for Cancer Research
R01-CA-22677 Schreiber	Pathobiology of Myeloma and Anti-Idiotypic Immunity University of Chicago
R01-CA-22786 Bankert	Receptor Dynamics and Normal or Tumor Cell Function Roswell Park Memorial Institute
R01-CA-22845 Scott	Immune Response to Modified Self and Tumor Antigens Duke University
R01-CA-23025 Hildemann	Comparative Transplantation Immunogenetics University of California, Los Angeles

R01-CA-23262 Bollum	DNA Polymerases in Normal and Leukemic Lymphoid Cells U.S. Uniformed Services Univ. Hlth. Sci.
R01-CA-23354 Koren	Natural Tumor Cell Killing in Humans Duke University
R01-CA-23593 Sanderson	Response of Leukocytes to Human Tumor Cells University of Colorado Health Sciences Center
R01-CA-24338 Fu	In Vitro Studies of Normal and Neoplastic Lymphocytes Rockefeller University
R01-CA-24431 Benjamin	Cellular and Structural Basis of Immunological Tolerance University of Virginia
R01-CA-24436 Wofsy	Receptor Function in Lymphocyte Differentiation University of California, Berkeley
R01-CA-24442 Sercarz	Chemical Basis for Receptor Recognition of Lysozymes University of California, Los Angeles
R01-CA-24450 Redelman	T-Cell Receptor and Effector Molecules University of California, San Diego
R01-CA-24472 Basch	Development of Thymic Lymphocytes New York University
R01-CA-24607 Engleman	HLA Restricted Suppressor T Cells of Mixed Lymphocytes Stanford University
R01-CA-25054 Mullen	Cellular Mechanisms Regulating Antibody Production University of Missouri, Columbia
R01-CA-25253 Bankert	Immunoregulatory Network Probed by Cell Hybridization Roswell Park Memorial Institute
R01-CA-25416 Koo	Immunogenetics of NK-1 Plus Natural Killer Cells Sloan Kettering Institute for Cancer Research
R01-CA-25583 Lopez	Cell Mediated Immunity in Mouse Mammary Tumor Models University of Miami
R01-CA-25612 Plate	Immunological Effects on Tumor Growth and Rejection Rush-Presbyterian-St. Luke's Medical Center
R01-CA-25738 Scheid	T Cell Differentiation: Molecular Mechanisms Sloan Kettering Institute for Cancer Research
P01-CA-25803 Klinman	Control of Normal and Abnormal Cell Development Scripps Clinic and Research Foundation
R01-CA-26084 Hale	Interactions Between Tumor Cells and T Lymphocytes Wake Forest University

R01-CA-26297 McKean	Primary Structure of MHC I Region Associated Antigens Mayo Foundation
R01-CA-26467 Stout	Effector and Suppressor Mechanisms of Tumor Immunity Brandeis University
R01-CA-26480 Dray	Antitumor Activity of Tumor-Bearer Lymphoid Cells University of Illinois Medical Center
R01-CA-26512 Plate	Rush-Presbyterian-St. Luke's Medical Center
R01-CA-26695 Cantor	Antigen-Specific T-Cell Clones: Generation and Analysis Sidney Farber Cancer Institute
R01-CA-26713 Clark	Genetics and Regulation of Cell-Mediated Cytotoxicity University of Washington
R23-CA-27552 Green	Cytotoxic T Cells to Syngeneic MULV+ Tumors Fred Hutchinson Cancer Research Center
R01-CA-27691 Ozer	T Cell Subset Immunoregulation--Myeloma and CL Leukemia Roswell Park Memorial Institute
R01-CA-27854 Bell	Cell Surface Carbohydrate and Lymphocyte Interactions University of Rochester
R01-CA-28099 Amos	F.A.C.S. of Immunologic Components Duke University
R01-CA-28196 Hudig	Proteinases of Human Natural Killer Cells University of California, San Diego
R01-CA-28332 Lord	In Situ Anti-Tumor Immunity and Effects of Radiation University of Rochester
R01-CA-28533 Russell	Mechanisms of Tumor Destruction by Immune Effectors Washington University
R01-CA-28708 Rohrer	Immunoregulation of Myeloma Cell Differentiation University of South Alabama
P01-CA-28900 Eisen	Control of Antigen-Specific T Cell Responses Massachusetts Institute of Technology
R01-CA-28936 Haynes	Immunoregulation in Autoimmunity and Malignant Disease Duke University
R01-CA-29282 Waksal	Prothymocyte Maturation and Function Tufts University
P01-CA-29606 Gershon	Immunoregulation--T Cells and Their Products Yale University



R01-CA-29635 Pauly	Analysis of Human T Lymphocyte Subsets Grown In Vitro Roswell Park Memorial Institute
R23-CA-29803 Macphail	Cytotoxic Cell Responses to Non-H2 Antigens Sloan Kettering Institute for Cancer Research
R01-CA-30147 Gottlieb	Genetic Markers, Leukemogenesis and Thymic Function University of Texas, Austin
R23-CA-30183 Klimpel	Bone Marrow Cytotoxic Precursor T Cells University of Texas Medical Branch, Galveston
R23-CA-30188 Scheffel	Autorecognition and Immunoreactivity Marquette University
R01-CA-30280 Weisbart	T-Lymphocyte Regulated Tumor Cell Killing by Neutrophils University of California, Los Angeles
R01-CA-30972 Bockman	Marrow Prostaglandins and T-Cell Differentiation Sloan Kettering Institute for Cancer Research
R01-CA-31534 Tucker	Isotype Switching in a Neoplastic B Cell Model, BCL1 University of Texas Health Sciences Ctr., Dallas
R01-CA-31564 Yates	Role of Glycolipids in Glioma Resistance to Cytolysis Ohio State University
R23-CA-31591 Yen	Regulation of Human B Cell Proliferation University of Iowa
R01-CA-31687 Donnenberg	Mechanisms of Lymphocyte Colony Formation Johns Hopkins University
R01-CA-31918 Fanger	Antibody Dependent Cell Cytotoxicity Reaction Mechanism Dartmouth College
R01-CA-31982 Ballas	Cytotoxic T Lymphocytes: Mechanisms of Generation University of Iowa
R01-CA-32018 Perry	T Subset Interactions in Specific Tumor Immunotherapy Emory University
R23-CA-32133 Susskind	Regulatory Mechanisms in Cell-Mediated Immunity Sloan Kettering Institute for Cancer Research
R01-CA-32277 Lynch	Fc Receptor-Bearing T Lymphocytes in Murine Myeloma University of Iowa
R23-CA-32593 Giorgi	Cytotoxic T Lymphocyte Lines Murine Plasmacytomas Massachusetts General Hospital
R01-CA-32685 Sondel	The Immunobiology of Human Antileukemic Lymphocytes University of Wisconsin, Madison

R01-CA-32739 Levy	Human T Lymphocyte Antigens and Their Genes Stanford University
R23-CA-32757 Hamilton	Mechanisms of Minor-H Antigen GVHD University of Washington
R01-CA-32801 Thorbecke	Immune Responses to Chemically-Induced Tumors in the CHI New York University
R01-CA-32841 Gooding	Effector Mechanisms in Rejection of SV40-Induced Tumors Emory University
R01-CA-33058 Kumar	Studies of Isolated Marrow-Dependent (M) Cells University of Texas Health Sciences Ctr., Dallas
R01-CA-33104 Basch	Somatic Cell Genetic Analysis of T-Cell Differentiation New York University
R01-CA-33529 Cullen	Processing of Ia Molecules in B Cells and Macrophages Washington University
R01-CA-33556 Smith	Autologous Mixed Lymphocyte Interactions Thomas Jefferson University
R23-CA-33588 Baum	Role of C-Reactive Protein in the NK Response Rush-Presbyterian-St. Luke's Medical Center
R01-CA-33654 Huttfleth	Functions of Atypical Lymphocytes University of Florida
R01-CA-34105 Brown	Immunoregulation: Idiotype Networks and Clonal Dominance St. Jude Children's Research Hospital
R01-CA-34106 Hayes	Murine T Cell Ia Antigens University of Wisconsin, Madison
R01-CA-34107 Flynn	Mineral Elements in the Generation of Cytotoxic T Cells Cleveland Clinic Foundation
R01-CA-34109 Waltenbaugh	Helper Cells/Factors from Nonresponders Northwestern University
R01-CA-34111 Miller	Post-Thymic T Cell Lineage Analysis George Washington University
R01-CA-34129 Burakoff	Regulation of Human and Murine Cytolytic T Lymphocytes Sidney Farber Cancer Institute

#### MONOCYTES AND MACROPHAGES

R01-CA-14113 Shin	Tumor Defense by Platelets and Macrophages Johns Hopkins University
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R01-CA-15236 Schreiber	Macrophage Recognition and Tumor Cell Interactions University of Pennsylvania
R01-CA-16652 Walker	Macrophage Functions in Tumorigenesis St. Jude Children's Research Hospital
R01-CA-16784 Adams	Tumoricidal Effects of Macrophages: Pathologic Study Duke University
R01-CA-19052 Moore	Development and Function of Activated Macrophages Sloan Kettering Institute for Cancer Research
R01-CA-20822 Colvin	Cell Interaction and the Clotting System Massachusetts General Hospital
R01-CA-22090 Nathan	Antitumor Action of Phagocytes and Lymphocytes Rockefeller University
R01-CA-24686 Morahan	Macrophage Extrinsic Activity Vs. Viruses Virginia Commonwealth University
R01-CA-25052 Niederhuber	Immune Responses In Vitro-H-2 (Ir) Locus Function University of Michigan
R01-CA-26824 Mantovani	Mononuclear Phagocytes in Human Ovarian Carcinoma Mario Negri Institute Pharmacologic Research
R01-CA-26846 Mussn	Mechanism of Human Monocyte Differentiation National Jewish Hospital and Research Center
R01-CA-26996 Fishman	Characterization and Functional Studies of "A" Cells St. Jude Children's Research Hospital
R01-CA-27523 Evans	Macrophages and Tumor Growth Jackson Laboratory
R01-CA-27639 O'Dorisio	Inhibition of Macrophage Tumor Cell Cytotoxicity Ohio State University
R01-CA-27694 Tompkins	Cytotoxic Macrophages Activation and Target Recognition University of Illinois, Urbana-Champaign
R23-CA-28935 Cameron	Macrophage Mediated Tumor Cytotoxicity Medical University of South Carolina
R01-CA-29266 Weiner	Characterization of Monocyte Subsets in Blood University of Florida
R23-CA-29333 Klykken	Metabolic Events Related to Macrophage Activation by MVE Virginia Commonwealth University
R01-CA-29336 Erickson	Macrophage-Mediated Cytotoxicity of Tumor Targets University of California, Davis

P01-CA-29589 Adams	Macrophage Activation: Development and Regulation Duke University
P01-CA-30198 Silverstein	Human Mononuclear Leukocytes in Cancer Rockefeller University
R23-CA-30631 Price	Targets of a Leukosis Virus Infection Trenton State College
R01-CA-31199 Russell	Macrophage-Mediated Injury Causing Tumor Regression University of Florida
R01-CA-31202 Russell	Monoclonal Antibody Depletion of Macrophages In Vivo University of Florida
R01-CA-31447 Zwilling	Prostaglandin Control of Macrophage Antitumor Activity Ohio State University
R01-CA-32551 Stanley	Hemopoietic Stem Cell Differentiation to Macrophages Yeshiva University
R01-CA-32898 Trinchieri	Differentiation and Function of Human Monocytes Wistar Institute of Anatomy and Biology
R23-CA-33003 Becker	Characterization of Human Macrophage Heterogeneity University of North Carolina, Chapel Hill
R01-CA-33225 Pelus	Regulation of Myeloid Progenitor Cell Differentiation Sloan Kettering Institute for Cancer Research
R01-CA-33558 Kim	Development and Function of Pulmonary Macrophages Sloan Kettering Institute for Cancer Research
R01-CA-33629 Kaplan	Differentiation and Anti-Tumor Activity of Macrophages University of Kentucky

#### MALIGNANCIES OF THE IMMUNE SYSTEM (LYMPHOMA/LEUKEMIA)

R01-CA-08975 Metzgar	Human Leukemia Associated Antigens Duke University
R01-CA-12779 Nowell	Leukocyte Regulatory Mechanisms University of Pennsylvania
R01-CA-13701 Murphy	Mechanisms of Immunity in Leukemia University of Michigan
R01-CA-15472 Eisen	Immunity to Myeloma Tumors Massachusetts Institute of Technology
R01-CA-17276 Tanigaki	Membrane Antigens from Normal and Leukemic Lymphocytes Roswell Park Memorial Institute

R01-CA-18602 Casper	Immunocompetent Cells in Acute Lymphocytic Leukemia Medical College of Wisconsin
R01-CA-20499 Edelson	Immunobiology of Cutaneous T Cell Lymphomas Columbia University
R01-CA-24950 Datta	A Thymus Determined Mechanism of Leukemia Resistance Tufts University
R01-CA-25097 Kersey	Differentiation of Immune System: Cell Surface Antigens University of Minnesota at Minneapolis
R01-CA-25369 Schlossman	Human Leukemia Antigens: Isolation and Characterization Sidney Farber Cancer Institute
R01-CA-25391 Dietz	Immunological Control of Dormant Leukemia Michigan Cancer Foundation
R01-CA-25613 Ross	Membrane Components of Normal and Leukemic Leukocytes University of North Carolina, Chapel Hill
R01-CA-25873 Humphreys	Membrane Proteins of Human Leukemias and Lymphomas University of Massachusetts Medical School
R01-CA-26369 Hauptman	T-MICG and N-MICG in Lymphoid Malignancy Thomas Jefferson University
R01-CA-27416 Mohanakumar	Characterization of New Human Ia and Leukemia Antigen Virginia Commonwealth University
R23-CA-27542 Fredericksen	T Cell Subsets and Marek's Disease Viral Oncogenesis New York University
R01-CA-27690 Koziner	Multiple Cell Marker Analysis in Hematopoietic Tumors Sloan Kettering Institute for Cancer Research
R01-CA-27826 Bach	Manipulation of Antitumor Immunity In Vitro University of Minnesota of Minneapolis-St. Paul
R01-CA-27942 Strober	Role of the Spleen in the Growth of a B Cell Leukemia Stanford University
R01-CA-28416 Rudders	Receptors for Immunoglobulin on Human Lymphoma-Leukemia New England Medical Center Hospital
R01-CA-28504 Chiao	T Cell Growth and Differentiation in Leukemia Sloan Kettering Institute for Cancer Research
R01-CA-28746 Fox	Cell Surface Antigens in Neoplastic and Autoimmune Disease Scripps Clinic and Research Foundation
R01-CA-29655 Mackenzie	Cytogenetic Studies of Human Myeloma University of California, Davis



R01-CA-29964 Haughton	UNC-CH Immunocytomas University of North Carolina, Chapel Hill
R01-CA-31479 Ford	Proliferation and Differentiation in Human Lymphoma University of Texas System Cancer Center
R01-CA-31685 Lebien	Differentiative Programs of Lymphoid Progenitor Cells University of Minnesota of Minneapolis-St. Paul
R01-CA-31792 Bennett	Immunobiology of Viral Leukemia and Its Treatment University of Texas Health Science Ctr., Dallas
R23-CA-32800 Zamkoff	Monocytes and the Immunodeficiency of Hodgkin's Disease Upstate Medical Center
R01-CA-32826 Macher	Glycosphingolipids in Oncogenesis and Differentiation University of California, San Francisco
R01-CA-33100 Chiao	Defects of AML Leukemia in Replication and Maturation Sloan Kettering Institute for Cancer Research
R23-CA-33127 Mangan	Regulation of Erythropoiesis in B Lymphocyte Neoplasms Montefiore Hospital

#### IMMUNE SURVEILLANCE

R01-CA-11898 Bigner	Etiology, Immunology and Biology of Brain Tumors Duke University
R01-CA-15988 Stutman	Immune Surveillance and Cancer Sloan Kettering Institute for Cancer Research
R01-CA-16136 Sell	The Role of Rabbit Lymphoid Cells in Tumor Immunity University of California, San Diego
R01-CA-19754 Cohn	Immunoselection and Cancer: A Problem in Evolution Salk Institute for Biological Studies
R01-CA-20408 Shultz	Immunodeficiency and Tumorigenesis Jackson Laboratory
R01-CA-20816 Gershwin	The Pathogenesis of Autoimmunity in New Zealand Mice University of California, Davis
R01-CA-20833 Trinchieri	Cell-Mediated Cytotoxicity in Humans Wistar Institute of Anatomy and Biology
P01-CA-21825 Grey	Self-Nonself Discrimination and Tumor Recognition National Jewish Hospital and Research Center
R01-CA-22517 Normann	Monocyte Function in Neoplasia University of Florida

R01-CA-23809 Saksela	Natural and Tissue-Specific Immunity to Human Neoplasms University of Helsinki
R01-CA-24497 McBride	Immunogenetic Analysis of Rous Sarcoma Development Baylor College of Medicine
R01-CA-24608 Grant	Tumor Immunity and Leukemia Harvard University
R01-CA-25250 Klein	Natural Killer Cells: Genetic Control and Role Caroline Institute
R01-CA-25641 Lo Buglio	Effect of Cancer on Human Monocyte Cytotoxic Mechanisms University of Michigan at Ann Arbor
R01-CA-25686 Sumaya	Epstein-Barr Virus: Effects on Human Lymphocytes University of Texas Hlth. Sci. Ctr., San Antonio
R01-CA-25917 Daynes	Cellular and Genetic Aspects of Antitumor Immunity University of Utah
R01-CA-26344 Weksler	Autologous Lymphocyte Reactions and Immune Surveillance Cornell University Medical Center
R01-CA-26752 Wigzell	Relevance and Functions of Natural Killer Cells University of Uppsala
R01-CA-26782 Kiessling	In Vivo Role of Natural Killer Cells Caroline Institute
R01-CA-26942 Fuji	H-2 Linked Resistance to Tumor: Effectors and Targets New York State Department of Health
R01-CA-27599 Williams	Genetic Control of Resistance and Immunity to P815 Northwestern University
R01-CA-28231 Carlson	H-2 Associated Natural Resistance Jackson Laboratory
R01-CA-28834 Dvorak	Basophil/Mast Cell Function in the Control of Cancer Beth Israel Hospital
R01-CA-29355 Blank	T-Cell Nonresponsiveness in Gross Virus-Infected Mice University of Pennsylvania
R01-CA-29910 Pattengale	Human NK Cells, Interferon(s) and Leukemia/Lymphoma University of Southern California
R01-CA-30115 Babcock	Immune Reactivity to Primary Sarcomas in Mice University of Texas Health Sciences Ctr., Houston
R01-CA-31344 Kay	Regulation of Human Natural Killer Lymphocyte Activity University of Virginia, Charlottesville

R01-CA-32576 Wheelock	Suppression of Established Leukemia Virus Infections Hannemann Med. Col. and Hospital of Philadelphia
R01-CA-32682 Gorelick	Natural Killer Cells in Metastatic Control Institute for Medical Research

#### IMMUNOTHERAPY IN ANIMAL MODELS

R01-CA-16642 North	Immunological Basis of Tumor Regression Trudeau Institute
R01-CA-27794 North	Mechanisms of Endotoxin-Induced Tumor Regression Trudeau Institute
R01-CA-29992 Pierpaoli	Prevention of Oncogenesis via Marrow Transplantation Foundation for Basic Biomedical Research
R01-CA-30303 Hunter	Selective Stimulation of Cell Mediated Cancer Immunity Emory University
R23-CA-30686 Jones	Ex Vivo Immunosorption as Tumor Therapy Sloan Kettering Institute for Cancer Research
R01-CA-31859 Kedar	Immunotherapy of Cancer with TCGF-Grown Cytotoxic Cells Hebrew University of Jerusalem
R01-CA-32045 Herd	Monoclonal Antibody Analysis and Therapy of B16 Melanoma Oberlin College
R23-CA-32109 Miller	Adsorbed Leukemic Sera Depress Cultured Blast Viability University of Minnesota of Minneapolis-St. Paul
R01-CA-33650 Altman	T Cells and Their Lymphokines in Cancer Immunotherapy Medical Biology Institute
N01-CB-84243 Thorn	Immunoprophylaxis of Bovine Lymphosarcoma University of Pennsylvania

#### BONE MARROW TRANSPLANTATION

R01-CA-20044 Winn	Transplantation Immunology Massachusetts General Hospital
R01-CA-28701 Beschornor	Chronic Graft-Versus-Host Disease in Radiation Chimera Johns Hopkins University
R01-CA-29592 Kahan	Active Specific Immunotherapy in Man: A Murine Model University of Texas Hlth. Sci. Ctr., Houston

## RESOURCES

N01-CB-23886      Facility for Supplying Immune-Related Cell Lines  
Melvin Cohn                      Salk Institute

N01-CB-15533      Resource Bank and Distribution Center for Cell Lines Useful in  
Research in Tumor Immunology  
Anita C. Weinblatt              American Type Culture Collection

# CONTRACT RESEARCH SUMMARY

Title: Resource Bank and Distribution Center for Cell Lines Useful in Research in Tumor Immunology

Principal Investigator:  
Performing Organization:  
City and State:

Dr. Anita C. Weinblatt  
AMERICAN TYPE CULTURE COLLECTION  
Rockville, MD

Contract Number: N01-CB-15533  
Starting Date: 9/28/81

Expiration Date: 9/27/86

Goal: To provide an efficient system for the acquisition, cataloging, storage and maintenance of cell lines which are capable of long term growth in vitro and are useful in tumor immunology research. Expert advice on culture and characteristics of all lines shipped will be offered to recipients.

Approach: The cell lines in the bank will be listed in a catalog, to be updated annually. New acquisitions will also be announced by means of newsletters. The cell lines in the bank will include, but not be limited to: B and T cell lines; lines useful in the study of macrophage/monocyte development; myelomas and their variants; cell lines useful in the study of immune effector mechanisms; and hybridomas. Lines will be shipped for a fee upon request. These lines will be screened for contamination with bacteria, fungi and mycoplasma; detailed characterizations will be performed.

Progress: Ampules of approximately 170 cell lines were transferred from the former contractor to ATCC shortly after this contract was negotiated. There were very few samples of each line so large volumes had to be grown up and be checked for species and contamination. Token freezes and distribution freezes were done next, or are in process, for the various lines. Of necessity this activity took precedence over acquisition of new lines but the acquisition phase should be well underway shortly. The first shipments of lines to customers started in January with shipment numbers increasing each succeeding month as inventory increased and advertisement of availability became more widespread. Through April 135 shipments have been made. This number is expected to change upward rapidly.

Project Officers: Dr. Bruce Maurer  
Ms. Judith Whalen  
Program: Immunology Section  
FY 82 Funds: 0



# CONTRACT RESEARCH SUMMARY

Title: Facility for Supplying Immune-related Cell Lines

Principal Investigator:	Dr. Melvin Cohn
Performing Organization:	THE SALK INSTITUTE
City and State:	San Diego, CA

Contract Number: N01-CB-23886

Starting Date: 6/26/72

Expiration Date: 11/30/81

Goal: To supply the scientific community with immune-related lines important in the study of tumor immunology and to increase the library of useful lines as they are characterized.

Approach: Since this is essentially a contract to supply cell lines, little experimental work is involved. However, the general thrust of the laboratory involved the isolation of immune-related lines of the thymus and bone marrow-derived lineages by the use of leukemia viruses, cell fusions, and chemical carcinogens. As new lines become available, they are introduced into the contract catalog.

Progress: The Cell Distribution Center continued to grow both in terms of the cell-line holdings, approximately 20 new cell lines being added to the catalog, and cell shipments with an average of 120 cell lines shipped per month.

There was an efficient transfer of samples of cell lines from the Salk inventory to the new contractor who won the competitive renewal of the cell bank, along with relevant documentation for each cell line. As appropriate, copies or originals of all pertinent correspondence were also sent to the new contractor.

Significance to Cancer Research: The lines in this contract are used by hundreds of laboratories for studies on tumor-specific antigens, antibody structure, immune-related cell functions, somatic genetics, and cell fusions. All of these subjects are major categories under the NCI Cancer Research Program, and the cell lines from this laboratory are key tools in these investigations.

Project Officer: Ms. Judith M. Whalen  
Program: Immunology Section  
FY 82 Funds: 0

# CONTRACT RESEARCH SUMMARY

Title: Immunoprophylaxis of Bovine Lymphosarcoma

Principal Investigator: Dr. Richard M. Thorn  
Performing Organization: UNIVERSITY OF PENNSYLVANIA  
City and State: Philadelphia, PA

Contract Number: N01-CB-84243  
Starting Date: 9/30/78                      Expiration Date: 1/29/82

Goal: Study the immunoprophylaxis of bovine lymphosarcoma through administration of BCG cell wall vaccine by intravenous injection as a possible guide for the control of analogous human disease.

Approach: Study the effect of the administration of BCG cell wall vaccine on the extent and rate of development of lymphosarcoma in cattle injected with bovine leukemia virus at a stage prior to the development of discernible evidence of disease.

Progress: The incidence of histologically confirmed lymphosarcoma following the second injection of BCG was 1/21 in the cattle receiving vaccine and 6/26 in the controls. This difference is not significant. BCG vaccination, as specified, did not have a statistically verifiable inhibitory effect. The mitogenic and spontaneous immune responses of cattle were not affected by BCG vaccination. BCG also caused no consistent changes in the hemograms or numbers of B or T lymphocytes or monocytes. The "course of disease" is at present based only on the palpation of lymph nodes and gross changes in blood lymphocytes number. Neither of these criterion is sufficiently unambiguous to allow detailed "staging" of the disease. Furthermore, once diagnosed, the disease progresses rapidly. Sequential measurements of immunologic and hematologic parameters did not reveal consistent changes that could be related to leukemia development. Some fluctuations in BLV antibody titer and in lymphocyte count appeared at the same time as lymphadenopathy, but no changes were detected that preceded the clinical signs of disease. The immunologic test results for BCG vaccinated cattle were indistinguishable from the control cattle. In summary, this BCG vaccination protocol did not reduce leukemia incidence significantly but the data are not conclusive because so few control animals died. Conversely, it was quite clear that BCG had no effect on any of the various specific and nonspecific immunologic and hematologic parameters measured. No parameters were found that preceded or coincided absolutely with the onset of the clinical signs of the disease.

Project Officer: Ms. Judith Whalen  
Program: Immunology Section  
FY 82 Funds: 0



## BREAST CANCER PROGRAM COORDINATING BRANCH

October 1, 1981 - September 30, 1982

### Description

The objectives of this extramural Program are to promote and support basic research in a variety of disciplines, as well as multidisciplinary research projects in the laboratory and in the clinic, with the aim of increasing our understanding and improving methods regarding the etiology, epidemiology, diagnosis, prognosis, treatment or prevention of breast cancer. These objectives are accomplished through the collaborative activities of the staff of the Branch and the members of the Breast Cancer Task Force Committee, an extramural advisory group. Through this interaction new and innovative research ideas are suggested, based on knowledge of ongoing research in breast cancer, and drawing upon the breadth of expertise represented, as well as upon information obtained at workshops and conferences in which the state-of-the-art on specific topics is presented. The staff of the Branch organizes the ideas into requests for investigator-initiated grant applications (RFA's) or program area announcements (PAA's), or requests for contract proposals (RFP's), depending upon the mechanism of funding considered most appropriate to achieve the goals of the project.

The Branch is organized into four Programs or Sections, namely, Diagnosis, Epidemiology, Experimental Biology, and Therapeutics, with a fifth section on Information. The grants and contracts managed by each of the four Programs have been categorized according to area of scientific emphasis and are presented at the end of this summary.

### Accomplishments

The grants program in breast cancer has increased in scope, in number, and in funding. In the previous fiscal year the program included 88 grants for a total of \$9.9 million dollars; and in this year the number has increased to 108 for a total of approximately \$11.6 million. This includes 99 R01's - regular grants, competitive and renewals; 6 R23's new investigator awards; and 3 P01's - program project grants (one in experimental biology, one in epidemiology, and one in treatment). The increase in number of grants has occurred in all four Programs, reflecting the breadth of research interest in this area. Some of the new grants in each Program have developed through encouragement of investigators by staff to apply for support for new and relevant breast cancer projects. In addition the Epidemiology area has funded two new grants this year that were among 12 responses to their PAA on Genetic Susceptibility to Human Breast Cancer, originally issued in January, 1980 (first funding of any responses to this PAA). Investigator interest in this subject has continued to increase, although slowly, reflecting the complexity of the area and of the resources of cancer prone families. Major advances in molecular biology since the original issuance prompted the reissue of the PAA this year with additions emphasizing chromosomal DNA areas of focus. Epidemiology's RFA on

Correlation Between Microscopic Characteristics of Primary Breast Tumors and Subsequent Patient Survival resulted in the eventual funding of three grant responses; it has also stimulated thinking in the scientific community and the submission of several other grants related to the question raised by the RFA. This has also been true of recent workshops on minimal breast cancer, on monoclonal antibodies, and on interaction of risk factors. In the interests of a balanced program, there has also this year been some appropriate redistribution of grants among the four Programs, and this trend will be continued and increased.

Projects funded by contracts have continued to decline. In the previous fiscal year there were 51 funded for a total of \$1.2 million; this year (FY 1982) there were 21, of which only 8 received Program funds (total \$236,000). Those not funded but continuing were either completing their final year or received extensions without additional dollars. The contract receiving the most dollars is service in nature.

In terms of scientific focus, the Diagnosis Program is focussing on biological markers of preneoplasia and/or early neoplasia and of prognosis; on methods of detection, especially by new non-invasive procedures; on morphologic discriminants of early breast cancer and of its risk of becoming invasive cancer; and on immunodiagnosis. Current areas of focus for the Epidemiology Program are genetic susceptibility, the endocrinology of breast cancer etiology and progression, benign breast disease and the natural history of breast cancer, environmental risk factors, and risk factors of possible relevance to tumors aggressiveness. The Experimental Biology Program has several areas of major research focus -- factors in tumor induction, responses to and dependence on hormones, pathophysiology and relation to metastasis, cell surface proteins, interaction with other cell populations, prevention of neoplastic transformation, immunodiagnosis and immunoprevention, and growth and passage of cells in vitro. Present areas of focus in the Treatment Program are hormone receptors - physiology, methodology; and clinical application; biological markers of tumor burden and of prognosis; mechanisms of treatment action; animal models for preclinical testing; and experimental clinical investigation.

Our external advisory group, the Breast Cancer Task Force Committee has had a membership of 25 this year, representing a broad range of disciplines, including endocrinology, pathology, immunology, biology, biochemistry, epidemiology, biostatistics, clinical oncology, radiology, surgery, and other areas relevant to breast cancer. This group has met as a body for workshop and program participation and discussion, but has also functioned as two smaller Working Groups for the purpose of closer interdisciplinary exchange and discussion regarding possible new research initiatives; one of these Groups has an etiology emphasis, encompassing epidemiology and experimental biology, while the other has a more clinical focus and concentrates on the diagnosis and treatment areas. The Committee has had three meetings in FY 1982. One of these included a large half-day workshop, sponsored by Epidemiology, on Interaction of Risk Factors. This was particularly well attended and has subsequently stimulated considerable attention by researchers and new analyses in several data sets to explore possible interaction. The other meetings have similarly devoted a portion of the schedule to a scientific session for the entire group for presentations on areas of interest for possible avenues of new research. BCTF



attendance at the meetings has been excellent, with good interaction and esprit de corps. Audience attendance and participation at the program sessions is always high and includes many from NCI and other NIH areas, always a number of our contractors and grantees (who come voluntarily on their own funds), other basic and clinical investigators from universities and other research organizations in this country and abroad, some from commercial firms, and some lay persons. Reimbursement for participation goes, of course, only to Task Force members and invited speakers.

A number of new research initiatives were presented to the DCBD Board of Scientific Counselors in October, 1981, for concept review. The Board approved a total of eight new RFA's or RFP's for the Program: (1) one on evaluation of diaphonography, one to provide serum and tissue for the search for new markers, and one on development of new diagnostic modalities (for the Diagnosis Program); (2) one on cytogenetic studies of cells transformed by gene transfer (joint between the Experimental Biology and Epidemiology Programs); (3) two on a repository for human breast epithelium and on continuation of a bank for human breast tumor lines and breast cancer cell cultures (both for the Experimental Biology Program); (4) one on luteal phase defects and breast cancer risk (for the Epidemiology Program); and (5) one on clonogenic assays to predict sensitivity to specific chemotherapies (for the Treatment Program).

#### Staff Publications

Sears, M. E., and Olson, K. B.: Extramural Review of Clinical Response of Breast Cancer to Cytotoxic Chemotherapy. Cancer 46: 2928-2929, 1980.

Tormey, Douglass C., Gelman, Rebecca, Band, Pierre R., Sears, Mary E., Bauer, Madeline, Arseneau, James C. and Falkson, Geoffrey: A Prospective Evaluation of Chemohormonal Therapy Remission Maintenance in Advanced Breast Cancer. Breast Cancer Research and Treatment 1. Martinus Nijhoff Publishers, pp. 111-119, 1981.

Albores-Saavadra, J., Cruz-Ortiz, H., Alcantara-Vazques, A, and Henson, D.E.: Unusual Types of Gallbladder Carcinoma. A Report of 16 Cases. Arch. Pathol. Lab. Med. 105: 287-293, 1981.

Macartney, J.C., Henson, D.E. and Codling, B.W.: Quality Assurance in Anatomic Pathology. Am. J. Clin. Pathol. 75: 467-475, 1981.

Weisman, I.D., Bennett, L.H., Maxwell, L.R., Jr., and Henson, D.E.: Cancer Detection by NMR in the Living Animal. In Diehl, P., E. Fluch, and R. Kosfeld (Eds), NMR Basic Principles and Progress. New York, Springer-Verlag, Berlin Heidelberg, 1981, pp. 17-37.

Codling, B.W., Macartney, J.C. and Henson, D.E.: Control Esterno de Calidad en Histopatologia Biopatal. Clinica. 187-206, 1981.

Egan, M.L., and Henson, D.E.: Monoclonal Antibodies and Breast Cancer. JNCI 68: 338-340, 1982.

Henson, D.E. and Grímley, P.: Virus Infections of the Fetus in Principles of Obstetrics. Caplan, R.M. (Ed), Williams and Wilkins, Baltimore, 1982.

Henson, D.E. and Grimley, P.: Virus Infections of the Fetus in Principles of Obstetrics. Caplan, R.M. (Ed), Williams and Wilkins, Baltimore, 1982.

Henson, D.E.: Interests of the Breast Cancer Task Force of NCI in Aspiration Needle Biopsy of the Breast. Pathologist 82: 258-259, 1982.

Alonso de Ruiz, P., Albores-Saavedra, J., Henson, D.E., and Monroy, M.N.: Cytopathology of Precursor Lesions of Invasive Carcinoma of the Gallbladder. A Study of Bile Aspirated from Surgically Excised Gallbladders. Acta Cytologica 26: 144-152, 1982.

Anderson, Elizabeth P.: Perspectives on Breast Cancer In Preventive Medicine, A Continuing Education Course, Vol. 1, Program 8, Audio-Visual, Johns Hopkins University, School of Hygiene and Public Health, April, 1982.

### Diagnosis Section

This section is concerned with improving diagnosis and finding better ways to estimate prognosis in breast cancer. This includes early diagnosis, separation of non-neoplastic from early neoplastic lesions, staging, determinants of biological behavior and predicting prognosis and recurrence. Through grants and contracts the Section supports basic and applied research related to diagnosis. The following describes different projects in the area of diagnosis.

### Biological Markers:

Efforts to find markers for breast cancer continue to receive special attention. The importance of markers is in their many clinical uses. For instance, they might detect incipient cancer, confirm a clinical diagnosis, or stage a patient for therapy. They may prove useful for estimating prognosis or for predicting recurrence. The Section has awarded several grants and contracts in its search for biological markers.

Concerning prognosis, the Section supports longitudinal studies in breast cancer patients to determine whether changes in the levels of serum enzymes or other blood constituents can be used to predict recurrence. Although time consuming, these studies are especially valuable. Based on information from initial presurgical blood data, a complex multivariate model has been developed that successfully predicted nearly 80% of recurrences. In addition to malignant disease, we have turned to benign breast disease in an effort to identify new markers, especially those that may predict the subsequent development of cancer.

Other research projects are concerned with separating premalignant hyperplasia from non-premalignant hyperplasia. These studies involved the use of biochemical, morphologic or immunologic techniques in order to associate putative markers from biopsy material with malignancy. They should have great practical significance if specific markers can be identified that are easily measured on routine histologic sections. Results from these projects have shown that CEA is present in malignant lesions and in severe epithelial dysplasia, but only rarely in cases of fibrocystic disease, IgG has been found in a pericellular pattern in dysplasia while IgA predominates in benign lesions.

### Methods of Detection:

Studies have also been initiated regarding non-invasive methods of detection. One study is designed to test the feasibility of localizing breast lesions by gamma imaging techniques using radiolabeled steroidal or other compounds that bind specifically to estrogen receptors. Studies on diaphanography and nuclear magnetic resonance are being planned. One study was funded in which a scanning electron microscope is used to magnify the images of microcalcification obtained by low-dose mammography. The purpose of this investigation is to determine if early granular calcification indicates the presence of cancer even before its pattern can be discerned on routine film examination by a radiologist. Although it is impractical to use a scanning electron microscope routinely, the results of this study should tell us if the pattern of microcalcification is established early in the evolution of neoplasia and if it can be used for early diagnosis.

An important concern is any adverse effect of the diagnostic modalities, especially mammography. Three grants were recently awarded to study the effects of irradiation on mammary carcinogenesis in rats and DNA damage repair in mammary cells.

### Morphologic Discriminants:

Accurate diagnosis and morphologic assessment of breast cancer remains a challenging problem for the Section. The statistics regarding the incidence of breast cancer indicate the need for early and effective diagnostic evaluation. Evaluation includes an assessment of the probability of invasive cancer following hyperplasia or in situ cancer as well as histologic markers for the separation of borderline lesions.

In order to ascertain the risk of invasive cancer with in situ and dysplastic lesions, we have supported a histologic review of a large number of carefully followed women with an initial diagnosis of in situ carcinoma, atypical hyperplasia or other benign conditions. This work is of practical importance because it may improve diagnostic criteria that pathologists use to estimate the biologic behavior of these lesions.

Studies are also underway to define whether expression of blood group antigens, such as the ABO system and T-antigen, can be used to predict metastatic spread. It is also possible that these blood group antigens may be useful in differentiating hyperplasia from early malignancy. Results with the T-antigen indicate that the pattern of intracellular distribution may be important. T-antigen was found only along the cytoplasmic membrane in 19 of 22 benign breast lesions whereas a diffuse cytoplasmic distribution was seen in 17 of 22 cases of breast cancer. The carcinomas which did not display the T-antigen were the most poorly differentiated of the group.

### Immunodiagnosis:

The use of immunological methods for diagnosis and for estimating prognosis is another important area of study. In the past studies were usually directed at correlating prognosis with the extent of the inflammatory response to the tumor, but more recent studies have been directed toward measuring the functional

ability of these lymphocytes. For example, one contract has been assessing the prognostic significance of cell-mediated immune reactivity in standardized in vitro tests. The results suggest that the tumor does affect functional activity in regional lymph nodes, an observation that may have bearing on the metastatic potential of breast cancer. This field should continue to attract considerable research interest for there is little information concerning the function of intratumor leukocytes and their relation to any immune response on the part of the host.

Also skin tests are being evaluated as a possible diagnostic help. There is evidence that the Thomassen-Frendenreich (T) antigen which is a carbohydrate precursor antigen for the MN blood group system is specifically associated with adenocarcinomas of the breast. Preliminary data indicate that breast cancer patients and a small number of patients with benign disease show both humoral and cell-mediated immune reaction to the T-antigen. As part of a prospective study, these antigens are being measured in patients without known breast cancer and in patients who will have a breast biopsy for suspected malignancy.

#### Other Activities:

A full-day workshop on "Monoclonal Antibodies in Breast Cancer" was held at the National Cancer Institute. A summary of the workshop was published in the Journal of the National Cancer Institute.

#### Epidemiology Section

This Section of the Breast Cancer Program encompasses research related to the epidemiology, etiology, and potential prevention of breast cancer. Twenty grants and seven contracts are categorized under eight different areas of focus in our studies. Five of these represent more than one category, since the categories are interrelated; one of these five is a program project grant with a broad, multidisciplinary approach to the etiology, epidemiology, and natural history of breast cancer. Five of the seven contracts are terminating in Fiscal Year 1981; one of the other two ongoing contracts is being extended without funds and will terminate in FY 1982. Ten new grants have been added to the program this year; two of these are responses to a particular Program Area Announcement and a third is related to a specific Request for Applications.

In the area of genetic susceptibility to breast cancer, one grant, following up our previous contract-funded studies by this same investigator, is extending research stemming from her very significant discovery that breast cancer susceptibility appears to be linked, in some but not in all breast cancer prone families, to the locus for glutamine pyruvate transaminase (GPT-1), a marker gene provisionally located on chromosome 10. This evidence for at least one gene for breast cancer susceptibility is a major breakthrough that has opened up new areas of research, including their continuing attempt to localize other genes for susceptibility through similar linkage analysis, as well as the search for the phenotypic expression of such a gene. Based on this finding, we issued in 1980 a Program Area Announcement on "Research Related to Genetic Susceptibility to Human Breast Cancer," and two new grants in response to this PAA have been funded this year. This will begin the critical process of exploring this and



other possible genetic linkages in different breast cancer families and in other geographic areas. Also, significant advances have recently been made in the study of DNA polymorphisms and related areas of molecular biology; this PAA was therefore reissued this year with three additions emphasizing chromosomal DNA areas of attention. Another grant related to genetic susceptibility is exploring hormone metabolism patterns associated with familial high risk.

The endocrinology of breast cancer etiology and progression has continued to be explored through a variety of studies: a) examining exogenous estrogens as a risk factor; b) defining endocrine events at the time of first pregnancy in young and in older women; c) exploring epidemiologic risk factors in relation to steroid hormone receptors and hormone binding globulin and examining these in relation to prognosis; d) studying hormone metabolism associated with familial breast cancer; e) searching for possible relationships between breast cancer and prior thyroid diseases; and f) assessing the carcinostatic effect of the pineal gland on DMBA, rat mammary tumors.

Program interest in the natural history of breast cancer has led to studies on the epidemiology of benign breast disease(s); a particular focus is the epidemiology of benign lesions most prone to progress to breast cancer. Incidence of benign breast disease is also being examined in ethnic subsets of the population known to differ in their risk of breast cancer, and interesting differences in breast histology are being observed in low risk *vs.* high risk women; in high risk women more abundant lobular tissue seems to persist with advancing age, providing more tissue at risk for carcinogenesis.

Concern about environmental risk factors has continued; attention has been focused not only on exogenous steroid hormones, but also on other possible carcinogens, including hair dyes and certain prescription drugs, and particularly on the possible role of diet. Special emphasis on cholesterol and lipids has been timely in terms of present interest in a possible negative correlation between risk for cardiovascular disease and risk for cancer; one ongoing contract is exploring differences between breast cancer cases and controls in lipid and/or cholesterol levels and metabolism. Another emphasis is on retinoids and vitamin A; the results with animal and *in vitro* studies have stimulated considerable interest in the potential of these substances as possible chemopreventive agents in human breast cancer. This was keenly discussed after a presentation to the Breast Cancer Task Force Committee in January of this year. One new grant in this area has been funded this year, and further activity on this topic is planned. Emphasis has been placed on possible mechanisms and rationale for dietary effects. The effect of obesity and of weight loss on steroid hormone metabolism is being determined. Dietary fat is being examined in relation to physical chemical properties of the cell membrane, immune responsiveness, and tumor cell susceptibility to cytotoxicity. In addition, carcinogens and mutagens that may particularly impinge on breast ductal epithelium are being determined from examination of breast fluids. Following our working session on Diet and Breast Cancer Risk in April, 1981, it has been concluded that some stimulus toward development of improved methodologies for dietary studies would be worthwhile and should probably be implemented.

Major interest has also focussed on risk factors of possible relevance to tumor aggressiveness and hence to prognosis. Our 1980 RFA on "Correlation Between Microscopic Characteristics of Primary Breast Tumors and Subsequent Patient



Survival" elicited a number of interesting responses, of which three were eventually funded; these and other studies are addressing specifics of epidemiology and of the morphology, biochemistry, immunology, and immunohistochemistry of breast tumors in relation to prognosis and survival. These include attention to viral antigens in relation to breast cancer risk and to prognosis, and exciting findings seem to be centering around, especially, antigens in human breast cancer related to the glycoproteins of mouse mammary tumor virus. Induction of breast fibroadenoma by human adenovirus is also being studied. The possible hormonal regulation of collagen metabolism is being explored in relation to metastatic potential of breast tumors.

High priority areas for future research, based on discussions in the Epidemiology - Experimental Biology Working Group of the Breast Cancer Task Force Committee, were presented in October, 1981, to the Board of Scientific Counselors, DCBD, for concept review. The Board unanimously approved the concept of new research emphases on "Cytogenetic Studies of Neoplastic Transformation of Mammary Cells and Mammary Tumors Through Chromosome- or DNA-Mediated Gene Transfer," and on "Luteal Phase Defects and Breast Cancer Risk". The latter is a follow-up of our very successful workshop on this topic held in December, 1980. It is anticipated that, as soon as feasible, the first of these will be issued (joint between Experimental Biology and Epidemiology) as a Program Area Announcement, while the second will be put forth as a specific Request for (Grant) Applications. In addition, a workshop this year on "Interaction Among Risk Factors" attracted considerable attention and has subsequently stimulated new thought and new data analyses exploring possible interaction in several different data sets. Another area of interest for possible future research emphasis is the epidemiology of breast cancer in certain, underexplored ethnic subsets of the population.

Following our working session in December, 1980, on Exogenous Menopausal Estrogens and Breast Cancer Risk, the Epidemiology Working Group, BCTF, set up an ad hoc subcommittee to organize a collaborative analysis of existent case-control data on this topic, in the hope of explaining some of the apparent inconsistencies among the findings from various studies. In the ensuing correspondence with investigators, all who are known to us to have data on this subject have agreed to participate in this collaborative project, which is now well under way. The approach is not to pool data but to determine maximum areas of commonality in the data collected with the aim of applying a standardized analysis to the various data sets. In this way, hopefully inferences can be pooled and the evidence to date clarified.

The overall emphasis of the Epidemiology Program is not simply statistical, descriptive epidemiology, but rather a broad, multidisciplinary approach to the exploration of possible etiologic mechanisms and the risk factors influencing these. The effort is consistently toward a deeper look at epidemiologic observations. The importance of multidisciplinary interaction continues to be emphasized. Program has fruitful exchange with staff from other Divisions of NCI and has maintained a continuing relationship with the Diet, Nutrition, and Cancer Program (DNCP); the Section Chief serves a member of the NCI Nutrition Working Group, and represents DCBD on that group and in its interaction with the Nutrition Subcommittee of the National Cancer Advisory Board.

## Experimental Biology Section

This section continues to maintain the largest number of investigator initiated research projects consisting of a variety of approaches to understanding basic biology. In addition, one contract continues to support a project that is a repository and distribution facility for animal tumor and human breast cell cultures, and antibodies to human and rat alpha-lactalbumin and to a variety of human and animal collagens and procollagens. Since the largest number of grants have been assigned to this Section, a larger number of funded grants has resulted, reflecting investigator emphasis on basic biology. There were 42 grants and one contract in the program for Fiscal Year 1982, including one program project grant and two new investigator awards.

The funded studies have been categorized into nine research areas: Factors in the Induction of Mammary Tumors; Growth Passage and Characterization of Normal Mammary Epithelial Cells; Mammary Tumor Responses to and Dependence on Hormones; Interaction of Neoplastic Mammary Cells with other Cell Populations; Cell Surface Proteins in Relation to Metastasis; Prevention of Neoplastic Transformation; Pathophysiology of the Solid Mammary Carcinoma and its Relation to Metastasis; Immunodiagnosis and Immunoprevention of Cancer Growth and Progression; and one Service, Support and Resource contract. Some of these grants are listed in two or more different categories because of overlapping nature of the research. This listing of funded studies appends this report, together with a research summary on contract CB-74175, including information on the goal, approaches and progress.

As a result of discussions held by the Experimental Biology Working Group of the Breast Cancer Task Force (BCTF) in 1981, projects calling for future research were presented to the Board of Scientific Counselors, DCBD, meeting held in October 1981, for concept review. The Board approved the concept of an RFA (joint with Epidemiology) on "Cytogenetic Studies of Neoplastic Transformation of Mammary Cells and Mammary Tumors Through Chromosome- or DNA-Mediated Gene Transfer" a concept they considered sound, timely and of much basic interest. The Board also approved the concept of an RFP for continuation of the "Animal and Human Mammary Tumors and Human Breast Cancer Cell Cultures Bank Facility" and one for "A Central Repository for Human Breast Epithelium from Non-Cancer Patients," possibly with combination of these two goals. Other future research under discussion includes preparation of antibodies directed against steroid hormone receptors, development of a reliable "in vitro" assay for neoplastic cell transformation, identification of chromosome carriers of transforming genes and modifications resulting from experimental carcinogenesis.

## Therapeutic Section

The treatment portion of the Breast Cancer Program is primarily concerned with developing new knowledge about tumor biology which can be applied to the optimal selection and utilization of various therapeutic modalities.

Eight contracts and twenty-seven grants are listed under five major research areas. Two contracts were terminated in Fiscal Year 1982 and five were confined to clinical follow-up observations.

For more than a decade the program has been deeply involved with steroid receptor technology and interpretation as a means of predicting hormone dependency of breast cancer tissue. Five grant supported studies are directed toward elucidating the physiologic activity of the steroid receptors. With the trend toward receptor assays being performed on all patients with primary and metastatic breast cancer, there is need for procedures that are simpler and more reproducible than the standard methods. There also is need for methods that will demonstrate the histologic distribution of the receptor proteins. Two grant supported projects and two contracts have these objectives. An evaluation oriented contract funded from the Office of the Director, NIH, is sampling the impact on clinical management of breast cancer produced by the application of steroid receptor data.

The search for biological markers in breast cancer patients continues. The Treatment Program has participated in the establishment for a bank of serum specimens from which qualified investigators may draw panels of coded specimens to test their purported markers. Two medical centers are processing serum samples from 1) asymptomatic women, 2) women with benign breast disease and 3) patients with breast cancer. The samples are stored in a facility where they are coded and held for distribution to the investigators. The Breast Cancer Data Center is heavily involved with the biomarkers project, classifying all specimens, selecting panels for distribution and analyzing the test results.

Research studies in the biomarker area are being carried out by two grantees and two contractors. Dr. Tormey is testing CEA and FHAP as breast cancer management parameters, Dr. Dao is studying sulfotransferase and sialyltransferase enzymes as clinical prognostic markers and Dr. Hilf is measuring a panel of tumor isoenzymes as potential prognosticators of response to chemotherapy.

A group of ten grant supported projects deals with the mechanisms by which chemical and endocrine treatments cause alterations in breast tumor growth. They explore specific tumor, host or treatment factors such as cytokinetics, immunological parameters, endocrine profiles during cytotoxic therapy, and intratumor steroid metabolism.

Animal models for preclinical treatment testing are the major thrust for two grant studies, a rat mammary carcinoma system for c. parvum and canine breast cancers for envelope glycoprotein 55. A third grantee is testing an *in vitro* system for predicting the effectiveness of chemotherapeutic drugs. Three further grant supported projects relate to biologic behavior of breast tumor cells concerning osteotropism, heterogeneity and mechanisms of invasion and extravasation.

The final category is clinical investigation with three contracts and two grants. Two of the contract supported studies are involved with systemic therapy, adjuvant to local therapy, for patients with cancer localized to the breast and axillary nodes. The third tests endocrine suppressive therapy against advanced breast cancer. The adjuvant studies have completed patient accrual and the courses of treatment and are in the long term follow-up phase. The advanced breast cancer project has also finished patient enrollment but continues with in depth endocrine studies. One of the adjuvant studies showed an advantage for patients with ER-positive cancers who received the anti-estrogen Tamoxifen in addition to CMF. The investigators have developed further treatment protocols and the



new project will be grant supported. The other grantee has collected extensive clinical and laboratory data on more than 800 breast cancer patients in a search for reliable prognostic indicators for recurrence and survival.

As an outgrowth of the Workshop in 1981 on Clonogenic Assays and Chemotherapy Sensitivity, an RFA is in preparation. It has been approved in concept by the Board of Scientific Counselors of DCBD.

### Information Section

The significance of this Section's program is the facilitation of access to comprehensive and timely information concerning breast cancer to enhance the quality of program formulation by staff and consultants. Responsibilities of the Section include the production monthly of Intercom, a publication which provides a bibliography of titles and journal references selected from the current periodical literature on research in breast cancer biology, epidemiology, diagnosis and treatment; a list of meetings and conferences related to the disease; and abstracts of presentations made at Workshops. The publication is produced by manual data entry and is sent to approximately 1900 investigators and institutions; about one-quarter of these are foreign. The Section has also made preparations for general distribution of Intracom, a publication which provides comprehensive coverage of breast cancer research, with abstracts, and includes coverage of grant and contract activity funded by any source, foreign or domestic. A much larger publication than Intercom, its distribution so far has been restricted to the Task Force Committee and Branch staff; any general distribution would be on microfiche to limit cost. Data acquisition is by a minicomputer programmed for automatic searching of MEDLARS and online data capture. In FY 1982, the production process has been developed to the point of producing a test tape which was sent to an outside source to verify successful conversion to hard copy by a Xerox 9700 system. This system can print both sides of a page, unlike the IBM 3800 system in the DCRT. From the same tape, this source can also produce microfiche which is free of format limitations imposed by the DCRT facilities. This completes the development and readies Intracom for possible global distribution, if this proves desirable, presumably merged with Intercom. Subsequently, however, the ICRDB has been forced to suspend the production of CANCERPROJ and the DCRT has eliminated the SPOUT utility, which necessitates some modification of the PLL software which was written in the Section to generate Intracom, and its production has been stopped. Thus Intracom becomes a demonstration project in which it was shown that:

It is possible to define a set of categories and formulate search logic which permits the production of a categorized compilation of breast cancer research generated by machine with very little human intervention at any stage from the initial search to final output. Consequently the compilation can be produced monthly and, based upon the ICRDB data bank, provides global coverage, with abstracts, of both published literature and grant/contract activity however funded. The same could be done for other organ sites.

External charges for publication on microfiche for a user group of 500 are \$7500-10,000 per year, including postage, and the incremental cost per user is \$12-15 per year.

Professional time required for the operations is one week per month.

The Section also conducts searches of machine-readable data bases to satisfy requests for information arising either intramurally or extramurally; in FY 1982, telecommunication software intended for transmission of search output and other text to remote sites has been developed to a prototype form in the dedicated mode (minicomputer occupied by no other task) to verify satisfactory operation of various technical features. Actually, the computer has other things to do and it would have to run in the multitasking mode (minicomputer doing more than one task simultaneously); the programming for the latter is a substantial undertaking. The minicomputer and the software which has been written for it represent a capability for automatic searching of MEDLARS and communication with the DCRT which can be uniquely useful to eliminate the long turnaround time which has hampered effective exploitation of automated information systems until now, since data captured online could be transmitted to users quickly by telecommunication (Wylbur mail for internal users).

A tracking system has been developed recently, which follows all grant applications from the time of initial receipt in the Branch to final notification of the applicants. It is a WYLBUR-based set of command procedures which govern all operations; hence, it can be operated by clerical personnel. ADP training activities have continued within the Branch.



BREAST CANCER TASK FORCE GRANT SUPPORTED STUDIES AND ONGOING CONTRACTS

DIAGNOSIS

I. Biophysical Tools for Detection and Diagnosis

RO1-CA-25836

Rational Design of Breast Tumor Localizing Agents  
Katzenellenbogen, John      University of Illinois

RO1-CA-28961

No-Dose Highly Magnified Mammograms to Aid Diagnosis  
Galkin, Benjamin      Thomas Jefferson University

RO1-CA-29993

Effects of X-Rays on Human Mammary Epithelial Cells  
Smith, Helene      University of California, Berkeley

RO1-CA-29940

Damage-Repair Studies Related to Mammography  
Han, Antun/Elkind, Mortimer      Argonne National Laboratory

RO1-CA-29781

Effects of Dose Rate on Rat Mammary Carcinogenesis  
Shellabarger, Claire      Brookhaven National Laboratory

RO1-CA-31624

Radioligands for Imaging Estrogen Responsive Tumors  
Hanson, Robert      Northeastern University, Boston

II. Immunodiagnosis

RO1-CA-32895

Monoclonal Antibody Hormone Dependent Breast Cancer  
Hendler, Freddy J.      University of Texas Health Science Center,  
Dallas

RO1-CA-25653 Antigen-Antibody Complexes in Breast Cancer

Chu, Tsann      Roswell Park Memorial Institute

RO1-CA-19083

T-Antigen in Human Cancer Detection  
Springer, Georg      Evanston Hospital

RO1-CA-29586

Immunocompetent Cells Infiltrating in Human Breast Cancer  
Carmack, Holmes      University of California, Los Angeles

RO1-CA-29601

Immunocompetent Cells Infiltrating in Human Breast Cancer  
Bhan, Atul      Harvard Medical School

RO1-CA-31302

Characterization of Immune Complexes in Human Breast Cancer  
Feller, William      Georgetown University

R23-CA-30370

Monoclonal Antibodies to Breast Cancer Immune Complexes  
Papsidero, Lawrence      Roswell Park Memorial Institute

NO1-CB-84228

Prognostic Significance of the Immunologic Response of Cells  
from Regional Lymph Nodes  
Cunningham-Rundles, Susanna      Sloan-Kettering

NO1-CB-84224

Detection of Immune Complexes in Sera of Patients with Breast Cancer  
Medof, Edward      University of Chicago

### III. Biologic Markers in Breast Cancer Preneoplasia

RO1-CA-32937

Markers of Mammary Cell Differentiation and Neoplasia  
Asch, Bonnie B.      Roswell Park Memorial Institute

RO1-CA-33038

Clinical Evaluation of a Blood Test for Breast Cancer  
Niedermeier, William      University of Alabama, Birmingham

RO1-CA-25574

Isozymes Specific to Human Breast Neoplasia  
Yang, Ning-Sun      Michigan Cancer Foundation

RO1-CA-30636

Human Immune Responses to Murine Mammary Tumor Virus  
Dion, Arnold      Institute for Medical Research

NO1-CB-84316

Biologic, Biochemical and Immunologic Characterization of "Pre-malignant" Human Mammary Epithelial Hyperplasias  
Jensen, Hanne      University of California, Berkeley

NO1-CB-74206

Longitudinal Studies in Biologic Markers in Breast Cancer Patients  
Schwartz, Morton      Memorial Hospital

NO1-CB-74086

Longitudinal Studies of Biologic Markers in Breast Cancer Patients  
Sussman, Howard      Stanford University

#### IV. Morphologic Discriminants

R23-CA-31755

In Vitro Studies on Mammary Neoplastic Progression  
Asch, Bonnie      Roswell Park Memorial Institute

R23-CA-28128

Tissue Blood Group Antigens and Carcinogenesis  
Howard, Donald      Maine Medical Center

#### EPIDEMIOLOGY

##### I. Genetic Aspects of Susceptibility to Breast Cancer; Hormone Metabolism Patterns Associated with Familial High Risk

RO1 CA-27632 (G)

Genetic Epidemiology of Breast Cancer in Families  
King, Mary-Claire      University of California, Berkeley

RO1 CA-29614 (G)

Genetics of Breast Cancer  
Anderson, David      University of Texas System Cancer Center

RO1 CA-30069 (G)

Estradiol Glucuronidation and Breast Cancer  
Zumoff, Barnett      Beth Israel Medical Center

RO1 CA-32280 (G)

Mendelian Factors in Human Breast Cancer  
Norum, Robert      Henry Ford Hospital

##### II. Steroid Hormones (Endogenous and Exogenous) and Their Contribution to Breast Cancer Risk; Hormonal Aspects of First Pregnancy; Epidemiologic Risk Factors in Relation to Levels of Steroid Hormone Receptors and of Estrogen-Binding $\beta$ -Globulin

NO1 CB-74099 (C)      Terminating 8-31-82

Estrogen Replacement after Pre-Menopausal Oophorectomy and Risk of Breast Cancer  
Slone, Dennis/Shapiro, Samuel      Boston University

NO1 CB-74101 (C)

Endocrine Events at the Time of First Pregnancy  
Preedy, John      Emory University

PO1 CA-13556 (G)

Epidemiology and Natural History of Breast Cancer  
Petrakis, Nicholas      University of California, San Francisco

RO1 CA-28720 (G)  
Breast Cancer Biology: Epidemiology and Prognosis  
Deubner, David      Duke University

RO1 CA-29622 (G)  
Breast Cancer, BBD, Hair Dyes and Menopausal Estrogens  
Nasca, Philip      New York State Department of Health

RO1 CA-30069 (G)  
Listed under I  
Zumoff, Barnett      Beth Israel Medical Center

III. Thyroid Function in Relation to Breast Cancer Risk

NO1 CB-84230 (C)      Terminating 9-29-82  
Relationship Between Thyroid Diseases and Breast Cancer  
Malooof, Farahe      Massachusetts General Hospital

IV. Pineal Gland and Nutrition Influences on Mammary Tumors

R23 CA-27653 (G)  
Pineal-Endocrine-Dietary Influence on Mammary Tumors  
Blask, David      University of Arizona

V. Epidemiology of Benign Breast Disease; Relation to Epidemiology and Natural History of Breast Cancer; Relation to Ethnicity and Risk of Breast Cancer

NO1 CB-74202 (C)      Terminated 1-14-82  
Epidemiology of Benign Breast Disease  
Spivey, Gary      University of California, Los Angeles

NO1 CB-84231 (C)  
Benign and Non-Invasive Breast Lesions in Populations at  
Different Risk for Breast Cancer  
Bartow, Sue      University of New Mexico

PO1 CA-13556 (G)  
Listed under II  
Petrakis, Nicholas      University of California, San Francisco

RO1 CA-26021 (G)  
Fibrocystic Breast Disease: Epidemiology and Histology  
Kelsey, Jennifer      Yale University

RO1 CA-29622 (G)  
Listed under II  
Nasca, Philip      New York State Department of Health

ROI CA-31698 (G)

Epidemiology of Breast Cancer after Fibroadenoma  
Dupont, William      Vanderbilt University

VI. Environmental Risk Factors for Breast Cancer (besides exogenous hormones)

- A. Diet (general; lipids and cholesterol; vitamins; other specific dietary factors; in relation to hormone levels and metabolism; in relation to properties of the cell membrane; in relation to immune responsiveness and tumor cell susceptibility to cytotoxicity; in relation to steroid receptor activation)

NO1-CB-84229 (C)      Terminated 3-29-82

Interrelationships Among Diet, Steroid Hormone Metabolism,  
and Human Breast Cancer  
Kirschner, Marvin      Newark Beth Israel Hospital

NO1-CB-84318 (C)      Terminating 9-30-82

Lipid Levels and Cholesterol Metabolism in Relation to Human  
Breast Cancer  
Papatestes, Angelos      Mount Sinai School of Medicine

ROI CA-30273 (G)

Dietary Fat Modulation of Mammary Tumorigenesis  
Erickson, Kent      University of California, Davis

ROI CA-30629 (G)

Promotion of Breast Cancer: Lipid-Hormone Interactions  
Cave, William      University of Rochester

ROI CA-33190 (G)

Dietary Vitamin A and Experimental Mammary Tumorigenesis  
Zile, Maija      Michigan State University

B. Hair Dyes; Drugs and Medications

ROI CA-29622 (G)

Listed under II  
Nasca, Philip      New York State Department of Health

ROI CA-32092 (G)

Role of Diazepam in Mammary Neoplasia  
Karmali, Rashida      Sloan Kettering Institute for Cancer Research

C. Possible Carcinogens and Mutagens in Breast Fluids

PO1 CA-13556 (G)

Listed under II  
Petrakis, Nicholas      University of California, San Francisco



VII. Correlation Between Breast Tumor Microscopic Characteristics and Patient Survival; Epidemiology, Morphology, Biochemistry, and Immunohistochemistry of Breast Tumors in Relation to Prognosis; Collagen Metabolism and its Possible Hormonal Regulation in Relation to Metastatic Potential

ROI-CA-28720 (G)

Listed under II

Deubner, David Duke University

ROI-CA-30335 (G)

Immunohistochemical Profile and Survival in Breast Cancer

Lee, Arthur New England Medical Center

(with Rosen, Peter Memorial Sloan-Kettering)

ROI CA-30342 (G)

RNA Virus-Related Antigen in Breast Cancer and Prognosis

Mesa-Tejada, Ricardo Columbia University

ROI CA-30215 (G)

Breast Cancer Prognosis by Tumor Differentiation

Sharkey, Francis Pennsylvania State University, Hershey

ROI CA-32240 (G)

Collagen Metabolism in Normal and Neoplastic Breast Tissue

Lewko, Walter University of Louisville

VIII. Viruses and Mammary Tumors; Antiviral Antibodies and Breast Cancer; Relation to Ethnicity, to Epidemiology, and to Prognosis

ROI CA-29711 (G)

Epidemiology and Etiology of Human Breast Cancer

Day, Noorbibi Memorial Sloan-Kettering

ROI CA-30342 (G)

Listed under VII

Mesa-Tejada, Ricardo Columbia University

ROI CA-31488 (G)

Induction of Breast Fibroadenoma by Human Adenovirus - 9

Topp, William Cold Spring Harbor Laboratory

EXPERIMENTAL BIOLOGY

I. Factors in the Induction of Mammary Tumors

ROI-CA-18664

Role of Prolactin in Mouse Mammary Tumorigenesis

Sinha, Yagya Scripps Clinic and Research Foundation

RO1-CA-27026  
Basis for Mammary Gland Susceptibility to Carcinogenesis  
Russo, Jose      Michigan Cancer Foundation

RO1-CA-27293  
Hormone Influences During Mammary Tumorigenesis  
Socher, Susan      Baylor College of Medicine

RO1-CA-20764  
Neoplastic Mammary Gland: Structure/Function  
Strum, Judy      University of Maryland

RO1-CA-25915  
The Radiobiology of Mouse Breast Preneoplasia  
Faulkin, Leslie J.      University of California, Davis

RO1-CA-31207  
Radioprotective Effect of Mammary Tumor Cell Grafts  
Scarantino, Charles      Bowman Gray School of Medicine

RO1-CA-21993  
Normal and Neoplastic Development of the Mammary Gland  
Dulbecco, Renato      The Salk Institute for Biological Studies

RO1-CA-22879  
Milk, Prolactin Binding and Thyroid in Breast Cancer  
Goodman, David      Albany Medical College of Union University

R23-CA-28999  
MMTV in Spontaneous and Carcinogen-Induced Tumors  
Pauley, Robert      University of Miami

RO1-CA-30036  
Mammary Metaplasia, Tumorigenesis and Chemoprevention  
Sorof, Sam      The Institute for Cancer Research, Fox Chase

RO1-CA-31774  
Estrogen Action in Normal Mammary Gland  
Haslam, Sandra      Michigan State University

RO1-CA-30570  
Modulation of Mammary Preneoplastic Progression  
Medina, Daniel      Baylor College of Medicine

RO1-CA-18175  
Endogenous Virus and Hormones in Mammary Cancer  
McGrath, Charles      Michigan Cancer Foundation

## II. Growth Passage and Characterization of Normal Mammary Epithelial Cells

PO1-CA-05388  
Biology of Mammary Neoplasia  
Nandi, Satyabrata      University of California, Berkeley

RO1-CA-24844

Characterization of Human Mammary Cells  
Stampfer, Martha      University of California, Berkeley

RO1-CA-29090

Characterization of MNU-Induced Mammary Cancers  
Grubbs, Clinton      Southern Research Institute, Birmingham

III. Mammary Tumor Response to and Dependence on Hormones

RO1-CA-21606

Role of Estrogen and other Hormones in Breast Cancer  
Butler, Barkley      Michigan Cancer Foundation

RO1-CA-20605

Mammary Cancer and Hormone Induced Responses  
Clark, James      Baylor College of Medicine

RO1-CA-30171

Steroid Hormones in Breast Cancer  
Hockberg, Richard      Yale University

RO1-CA-26869

Nuclear Estrogen Receptors in Breast Cancer  
Horwitz, Kathryn      University of Colorado Medical Center

RO1-CA-18664

Listed under I  
Sinha, Yagya      Scripps Clinic and Research Foundation

RO1-CA-27293

Listed under I  
Socher, Susan      Baylor College of Medicine

RO1-CA-28645

Vitamin B6 and Hormone Action in Uteri and Breast Cancer  
Wotiz, Herbert      Boston University School of Medicine

RO1-CA-28698

Perinatal Estradiol Influence on Mammary Development  
Warner, Marlene      Baylor College of Medicine

RO1-CA-28393

Neonatal Hormone Exposure and Mammary Tumorigenesis  
Talamantes, Frank      University of California, Santa Cruz

RO1-CA-18458

Fetal Exposure to Hormones and Mammary Carcinogenesis  
Boylan, Elizabeth      Queens College of CUNY

RO1-CA-16464

Iodinated Estrogens in Breast Cancer  
Caspi, Eliahu      Worcester Foundation for Experimental Biology

R01-CA-24687

Metabolism of Estrogens and Androgens by Breast Cancer Cells  
Macindoe, John      University of Iowa

R23-CA-29501

Estrogen Responsiveness in Human Breast Cancer  
Moore, Michael      Marshall University

IV. Interaction of Neoplastic Mammary Cells with other Cell Populations

R01-CA-25179

The Fibrotic Response to Human Breast Carcinoma  
Stern, Robert      University of California, San Francisco

R01-CA-20764

Listed under I  
Strum, Judy      University of Maryland

R01-CA-28366

Natural Site Preference in Mammary Cancer Biology  
Miller, Fred      Michigan Cancer Foundation

V. Cell Surface Proteins in Relation to Metastasis

R01-CA-19814

Metabolic Fate of Mammary Cell Surface Glycoconjugates  
Bernacki, Ralph      Roswell Park Memorial Institute

R01-CA-27909

Adhesive Interactions in Mammary Tumor Epithelial Cells  
Buck, Clayton      The Wistar Institute

R01-CA-31695

Sialoglycoproteins of a Metastatic Mammary Tumor  
Carraway, Kermit      University of Miami

R01-CA-19455

Use of Membranes to Assess Phenotypic Expression  
Ceriani, Robert      Children's Hospital Medical Center, Oakland

R01-CA-08418

Glycoproteins from Cancer Cells  
Codington, John      Massachusetts General Hospital

R01-CA-27314

Plasma Membrane and Metastasis in Rat Mammary Cancer  
Fairbanks, Grant      Worcester Foundation for Experimental Biology

R01-CA-26825

Antigen Specific NK Activity and Suppression in MMTV  
Lane, Mary-Ann L.      Sidney Farber Cancer Institute

R01-CA-25418

Murine Virus Cross-Reacting Antigen in Human Tissue  
Hackett, Adeline      University of California, Berkeley

R01-CA-28844

Mammary Carcinoma Metastasis  
Nicolson, Garth      University of Texas System Cancer Center

VI. Prevention of Neoplastic Transformation

R01-CA-30036

Listed under I  
Sorof, Sam      The Institute for Cancer Research, Fox Chase

VII. Pathophysiology of the Solid Mammary Carcinoma and its Relation to Metastasis

P01-CA-05388

Listed under II  
Nandi, Satyabrata      University of California, Berkeley

R01-CA-28735

Proteoglycans and Basal Structure and Function  
Bernfield, Merton      Stanford University

VIII. Immunodiagnosis and Immunoprevention of Cancer Growth and Progression

R01-CA-27437

Immune Reactivity and Mammary Neoplasia  
Heppner, Gloria      Michigan Cancer Foundation

R01-CA-30284

An Immunodiagnostic Assay for Breast Cancer Detection  
Gaffney, Edwin      The Pennsylvania State University

R01-CA-20286

Breast Neoplasia Diagnosis with Specific Antibodies  
Ceriani, Robert      Children's Hospital Medical Center, Oakland

IX. Service, Support and Resources

N01-CB-74175

Animal and Human Mammary Tumors and Human Cell Culture Bank Facility  
Bogden, Arthur E.      Mason Research Institute



## TREATMENT

### I. Hormone Receptors and Mammary Carcinoma

#### A. Physiologic Behavior

RO1-CA-22828

Fate of Steroid Hormones in Breast Tumor Cells

Brooks, Sam      Wayne State University School of Medicine

RO1-CA-26452

Anomalous Receptors in Breast Cancer

Panko, Walter      Baylor College of Medicine

RO1-CA-27470

Receptor Studies in Human Breast Cancer

Hollander, Vincent      Hospital for Joint Diseases

RO1-CA-30350

Evolution of Estrogen Receptor Status in Mammary Tumors

Kiang, David      University of Minnesota

RO1-CA-31895

Role of Epidermal Growth Factor Receptor in Breast Cancer

Schultz, Gregory      University of Louisville

#### B. Methodology

NO1-CB-14358

Prediction of Hormone Dependency in Human Breast Cancer

Jensen, Elwood      University of Chicago

NO1-CB-23862

Biochemical Mechanism of Endocrine-Induced Breast Cancer

Regression

McGuire, William      University of Texas, San Antonio

RO1-CA-23623

Monoclonal Antibodies to Localize Breast Cancer Receptors

Pertschuk, Louis      State University of New York, Brooklyn

RO1-CA-29971

Histochemical Methods for Receptors in Breast Cancer

Chamness, Gary      University of Texas, San Antonio

#### C. Evaluation of Clinical Application

NO1-CB-04338

Evaluation of the Impact of the Estrogen Receptor Assay on  
the Treatment of Human Breast Cancer

Thomas, David      Fred Hutchinson Cancer Research Center

## II. Biologic Markers in Breast Cancer

- N01-CB-43900  
Steroid Sulfation and Estrogen Binding in Human Breast Cancer  
Dao, Thomas      Health Research, Inc.
- N01-CB-74204  
Methods to Predict Chemotherapy Sensitivity  
Hilf, Russell      University of Rochester
- R01-CA-26935  
CEA and FHAP as Breast Cancer Management Parameters  
Tormey, Douglass      University of Wisconsin
- R01-CA-33246  
Pathophysiology of Sialyltransferase in Breast Cancer  
Dao, Thomas      Health Research, Inc.

## III. Mechanisms of Treatment Action

- R01-CA-02071  
Estrogen Metabolism and Action in Pregnancy and Cancer  
Levitz, Mortimer      New York University
- R01-CA-05197  
Endocrine Factors Influencing Tumor Growth in Man  
Pearson, Olof      Case Western Reserve University
- R01-CA-23079  
Steroid Sulfurylation Inhibitors as Antitumor Agents  
Horwitz, Jerome      Michigan Cancer Foundation
- R01-CA-24129  
Control of Breast Cancer by Serum Growth Factors  
Osborne, Kent      University of Texas, San Antonio
- R01-CA-25586  
Therapy-Induced Changes in Human Breast Cancer  
Panko, Walter      Baylor College of Medicine
- R01-CA-26004  
Therapy Effects on Tumor Kinetics and Immune Parameters  
Fisher, Bernard      University of Pittsburgh
- R01-CA-27440  
Aromatase Inhibitors and Control of Breast Cancer  
Brodie, Angela      University of Maryland
- R01-CA-29660  
Immunotherapy of Mammary Tumors  
Vaage, Jan      Roswell Park Memorial Institute

ROI-CA-30251

Hormone Priming and Chemotherapy in Primary Breast Cancer  
Osborne, Charles      University of Texas, San Antonio

ROI-CA-32713

Structure-Function Relationship of Anti-estrogens  
Jordan, V. Craig      University of Wisconsin

#### IV. Preclinical Testing: In Vitro and Animal Models

ROI-CA-26287

Canine Model for Human Breast Cancer Immunotherapy  
Hurvitz, Arthur      Animal Medical Center

ROI-CA-27419

Chemotherapeutic Consequences of Tumor Heterogeneity  
Heppner, Gloria      Michigan Cancer Foundation

ROI-CA-29006

C. parvum Therapy of Mammary Carcinoma Metastases  
Kreider, John      Pennsylvania State University, Hershey

ROI-CA-29537

Osteotropism of Mammary Carcinoma Metastasis  
Mundy, Gregory      University of Texas, San Antonio

ROI-CA-32071

Mechanisms of Tumor Cell Invasion and Extravasation  
Starkey, Jean      Washington State University

ROI-CA-32158

Effect of Chemotherapeutic Drugs on Human Mammary Cells  
Smith, Helene      University of California, Berkeley

#### V. Clinical Investigations

N01-CB-43990

Therapy for Stage II or Stage III Carcinoma of the Breast  
Hubay, Charles      Case Western Reserve University

N01-CB-53851

Suppression of Endocrine Function by Systemic Agents for  
Breast Cancer  
Santen, Richard      Pennsylvania State University

N01-CB-53917

Therapy of Patients with Stage II or Stage III Carcinoma of  
the Breast  
Scanlon, Edward      Evanston Hospital

P01-CA-16175

Breast Cancer Prognostic Study  
Brennan, Michael      Michigan Cancer Foundation

RO1-CA-30006

Chemotherapy and Anti-Estrogen Therapy in Breast Cancer

Hubay, Charles      Case Western Reserve University

CONTRACT RESEARCH SUMMARY

Title: Estrogen Replacement after Premenopausal Oophorectomy and Breast Cancer Risk

Principal Investigators:  
Performing Organization:  
City and State:

Dr. Dennis Slone, Dr. Samuel Shapiro  
Boston University Medical Center  
Cambridge, MA

Contract Number: N01-CB-74099

Starting Date: 9/1/77

Expiration Date: 8/31/82

Goal: To examine the hypothesis that exposure to female hormones, particularly estrogen replacement therapy, is related to an increased risk of breast cancer, either during use or after a latent interval.

Approach: An epidemiological study of the case-control type is being implemented as part of an ongoing, hospital-based, multicenter data collection system. Cases of breast cancer and potential controls, women with a variety of non-malignant conditions, are interviewed by trained nurse monitors in hospitals throughout the country. Information on lifetime drug use and relevant medical data are recorded on standard forms and transferred to computer files for analysis.

Progress: As of the end of February, 1982, about 3300 women with breast cancer had been interviewed. Over 2000 of these had been diagnosed within the year before admission. A detailed analysis of a file of 1610 women with breast cancer diagnosed within the six months before admission and 1606 control subjects is currently in progress. A total of 228 cases and 351 controls had used conjugated estrogens, giving a relative risk estimate adjusted for age and menopausal status of 0.9, with 95% confidence limits of 0.7 and 1.1. Among 132 cases and 306 controls who had premenopausal bilateral oophorectomy, 51 and 167, respectively, had used conjugated estrogens. The relative risk estimate for these women was 0.6, with 95% confidence limits of 0.4 and 1.0. Further results are not yet available. Eventually we expect to prepare a manuscript for publication based on these analyses.

Project Officers: Elizabeth P. Anderson, Ph.D., Robert Hoover, M.D.  
Program: Breast Cancer Epidemiology  
FY 82 Funds: 0



## CONTRACT RESEARCH SUMMARY

Title: Therapy for Stage II or Stage III Carcinoma of the Breast

Principal Investigator: Dr. Charles A. Hubay  
Performing Organization: Case Western Reserve University  
City and State: Cleveland, OH

Contract Number: N01-CB-43990

Starting Date: 6/16/74

Expiration Date: 6/15/83

Goal: This clinical trial was initiated in September 1974 to determine if combined chemotherapy (CMF), anti-estrogen therapy (Tamoxifen) and immunotherapy (BCG) following mastectomy in Stage II, III breast cancer would yield better results than when used later in the course of the disease when recurrence became manifest.

Approach: Patients under age 76 who show axillary nodes involved with metastases at the time of surgery were eligible for this study. Stratification, but not treatment selection, was on the basis of the presence or absence of estrogen receptor protein (ER) in the tumor. Random treatment and assignments were (1) cyclophosphamide, methotrexate, 5-fluorouracil (CMF); (2) cyclophosphamide, methotrexate, 5-fluorouracil, Tamoxifen (CMFT); and (3) CMFT for 12 months plus BCG for 12 months. Endpoint was the first evidence of treatment failure, i.e., the appearance of local recurrence or of distant tumor.

Progress: Entry into the study was closed in June 1979. Three hundred eighteen patients were enrolled in the study. Of these 76% had estrogen receptor (ER) positive tumors ( $\geq 3$  femtomoles/mg of protein). All patients have completed therapy. Of the treated patients, 111 have had treatment failure and 63 have died. Chemotherapy was not accompanied by significant myelosuppression. Hair loss was slight. Tamoxifen, the anti-estrogen agent used, was tolerated well, without serious side effects, although most patients experienced hot flashes while on the drug. One hundred eleven have had recurrence; 41 in the CMF group, 35 in the CMX + Tamoxifen and 35 in the CMG + Tamoxifen + BCG group. Statistical comparison for treatment failure between Stage II ER positive and ER negative patients is significant at the  $P < .0002$  level, with the latter group recurring more rapidly and ER positive patients have a prolonged survival ( $p < .05$ ). The patients with ER-positive tumors who receive Tamoxifen appear to have the most marked delay in recurrence when compared to those who receive CMF alone. Stratifying by nodal status, the additional of Tamoxifen to CMF appears to have more benefit in the  $\geq 3$  node group. ( $p < .055$ ). Similarly the addition of Tamoxifen to CMF appears to have more benefit for patients with tumors  $> 3.0$  cm. ( $p = .05$ ).

Project Officer: Mary E. Sears, M.D.  
Program: Breast Cancer Treatment  
FY 82 Funds: \$22,825

## CONTRACT RESEARCH SUMMARY

Title: Endocrine Events at the Time of First Pregnancy

Principal Investigator:	Dr. John R. K. Preedy
Performing Organization:	Emory University School of Medicine
City and State:	Atlanta, GA

Contract Number: N01-CB-74101

Starting Date: 8/1/77

Expiration Date: 7/31/82

Goal: It is proposed that the protective effect of an early first pregnancy against breast cancer is due to a change in the hormonal environment following that event. We proposed to investigate and characterize such a change.

Approach: Nulliparous subjects in the 18-22 age range who have not taken birth control pills, who have a history of normal regular menses, and who are planning pregnancy in the next 1-2 years will be selected (Group A). Similar subjects will be chosen who do not plan to become pregnant in the next 1-2 years (Group B). Similar subjects to the above but in the age range 30-40 would also be selected (Groups C and D). All groups would have the following carried out in the early follicular phase of the menstrual cycle: plasma gonadotropins, estrogens, progesterone, androgens; urine estrogens and androgens; plasma protein-steroid binding studies; perphenazine-prolactin stimulation test; LRH-LH stimulation test. Results from Groups A, B, C, and D would be compared by appropriate analysis of variance techniques to determine significant contrasts among four groups. The study design aims for 20 women in each group, each woman serving as her own "before" and "after" control.

Progress: The total number of subjects participating in the program is 122. Group A - 41. Initial study was done on all 41. Twenty-two have become pregnant, 4 of these have suffered miscarriage, and 18 have reached full term and delivered. As of March 31, 1982, the second and final study has been done on 14 of these, 2 are scheduled for restudy, and 2 have been lost to a second pregnancy before lactation was terminated and the restudy could be carried out. Group B - 29. All 29 underwent the initial study, and the second study has been made on all 20 allowed by the experimental design. Group C - 24. Initial study was made on all 24. To date, 12 have become pregnant; 1 of these has suffered a miscarriage, 10 have delivered, and 1 is still pregnant. Seven of the 10 have been restudied, 2 are scheduled for restudy, and 1 has been lost to a second pregnancy. Group D - 28. All 28 underwent the initial study; the second study has been made on 18 and is scheduled for the remaining 2. We expected to finish the study sampling this summer, and be able to complete laboratory and statistical analyses within another 9-10 months.

Project Officer: Elizabeth P. Anderson, Ph.D.  
Program: Breast Cancer Epidemiology  
FY 82 Funds: 0

# CONTRACT RESEARCH SUMMARY

Title: Therapy of Patients with Stage II or Stage III of the Breast

Principal Investigator: Dr. Edward F. Scanlon  
Performing Organization: Evanston Hospital  
City and State: Evanston, IL

Contract Number: N01-CB-53917

Starting Date: 6/30/75

Expiration Date: 6/29/83

Goal: To determine the efficacy of adjuvant chemotherapy and chemoimmunotherapy for patients who have had standard surgical intervention for Stage II or III carcinoma of the breast.

Approach: Patient accrual began in July 1975, and was completed in July 1979. A total of 194 patients were entered into the study during this time. Stratification and randomization were carried out along with a balancing for prognostic factors according to the following variables: primary tumor size, number of positive nodes, menopausal status, and unfavorable local signs. The statistical analysis involved sequential treatment assignment and was designed to provide the greatest balance among the three groups. Using this method, patients were originally assigned to one of three treatment schedules: Group I - Oral phenylalanine mustard (L-Pam), Group II - 5-Fluorouracil, cyclophosphamide and prednisone (CFP), and Group III - CFP plus BCG inoculations. Enrollment in the single agent treatment, Group I, was discontinued in November 1977 because of a disproportionately high recurrence rate in the group at that time. The 38 patients already enrolled in that group remained to finish their single agent treatment. New patients from that time were enrolled either in Group II or III.

Progress: Seventy-two patients have experienced recurrent disease (37%). Of the thirty-eight patients enrolled in Group I, 17 (45%) report recurrences. Thirty-one patients of the 78 patients in Group II present with recurrent disease (40%), and 24 patients of the 78 patients in Group III (31%) have recurrent disease. We continue to observe a steadily increasing recurrence rate in the polychemotherapy group, compared to the dropping recurrence rate in the single chemotherapy group. No statistically significant difference exists among the three groups with regard to disease-free interval. The two prognostic factors, tumor size ( $\geq 3$  cm) and nodal involvement ( $\geq 4$ ) continue to be statistically significant. The prognostic factor of presence of grave local signs is now emerging as statistically significant. The prognostic factor of presence of grave local signs is now emerging as statistically significant. Survival analysis indicates an unfavorable statistically significant difference for patients in Group I. Of the 38 patients in this group, 12 patients have expired of their disease. Of the 78 patients in Group II, 11 patients have died of their disease, and in Group III, five of the 78 patients have died of their disease. Two publications resulted from the project during the recent contract year.

Project Officer: Mary E. Sears, M.D.  
Program: Breast Cancer Treatment  
FY 82 Funds: \$24,435

## CONTRACT RESEARCH SUMMARY

Title: Evaluation of the Impact of the Estrogen Receptor Assay on the Treatment of Human Breast Cancer

Principal Investigator:	Dr. David B. Thomas
Performing Organization:	Fred Hutchinson Cancer Research Center
City and State:	Seattle, WA

Contract Number: N01-CB-04338

Starting Date: 9/1/80

Expiration Date: 9/30/82

Goal: To identify characteristics of women, their physicians and hospitals that distinguish individuals with breast cancer who have steroid hormone receptor assays performed on samples of their primary or recurrent tumor tissue, and to determine whether therapy for primary or recurrent disease is altered by the assay results.

Approach: Women with primary breast cancer in 1977-78 and 1980, and women in the former group who develop recurrent disease by the end of 1981 are identified through the Cancer Surveillance System (CSS) which is a population-based tumor registry that has been used to collect data on all newly diagnosed cancer cases in a 13-county area of Washington State since 1974. Descriptive information on all study subjects is being collected from the registry and this is supplemented by data on some women with disease onset in 1977-78 that were collected by personal interviews as part of a previous study. Information on steroid receptor assays is collected from medical records. Information on physicians is obtained from professional directories and a state survey of physicians; and data on hospitals are abstracted from similar sources. Histologic and clinical characteristics of the tumor and types of therapies given are obtained from CSS records and will be supplemented by querying physicians. Assay methods used and quality control procedures followed will be ascertained from laboratory directors.

Progress: Approximately 1200 female residents of King County who were diagnosed with breast cancer in 1977 or 1978 have been identified through the area SEER registry, and collection of information on steroid receptor assays, recurrences of disease, and therapy for recurrent tumors is nearing completion. About 1200 additional cases of breast cancer occurring in women in the 13-county registry catchment area in 1980 have also been identified, and registry personnel are currently obtaining information on steroid receptor assays and first course of therapy on these patients. Descriptive data on area hospitals and physicians have been obtained. A computerized data processing system for this study has been developed, and data are being coded, edited, and key-centered for analysis. Replies to special queries on about 500 cases are being received and data collection is complete on 90% of the study subjects. Coding has been completed for these cases and is complete for the cases being queried except for the additional information requested.

Project Officer: Mary E. Sears, M.D.  
Program: Breast Cancer Treatment  
FY 82 Funds: 0



# CONTRACT RESEARCH SUMMARY

Title: Steroid Sulfation and Estrogen Binding in Human Breast Cancer

Principal Investigator:	Dr. Thomas L. Dao
Performing Organization:	Health Research, Inc.
City and State:	Buffalo, NY

Contract Number: N01-CB-43900

Starting Date: 2/1/74

Expiration Date: 1/31/83

Goal: To determine the relationship of steroid sulfotransferase activity to estrogen receptor protein levels, to pathologic staging, and to risk of relapse.

Approach: Steroid sulfating enzyme activity and estrogen receptor (ER) levels have been assayed in breast tissue obtained from approximately 300 patients in three institutions. The evidence will be evaluated for or against correlation of the steroid sulfotransferase activity with risk for tumor-metastasizing potential and with histopathologic parameters. The predictive potentials of ER sulfation activity will be compared.

Progress: A total of 266 patients from three cooperating institutions were entered into the study between March 1974 and June 1976, inclusive. The average length of follow-up is now approximately 80 months. The data have been analyzed according to the following categories: DHEAS versus other prognostic indicators; E<sub>2</sub>S versus other prognostic indicators; ER versus other prognostic indicators; and the recurrence rate for categories of prognostic indicators. The prognostic indicators considered were: age at diagnosis; pathologic size of the tumor; histologically positive axillary nodes; and menopausal status. The synthesis of sulfotransferase was also evaluated for its correlation with age at diagnosis, tumor size, axillary nodal metastasis, and menopausal status. However, because of size, ER and sulfotransferase activities could not be measured in all tumors. The present analysis includes only those patients for whom both measurements were done. These data are being prepared for publication.

Project Officer: Mary E. Sears, M.D.  
Program: Breast Cancer Treatment  
FY 82 Funds: \$4,774



## CONTRACT RESEARCH SUMMARY

Title: Animal and Human Mammary Tumors and Human Cell Culture Bank Facility

Principal Investigator:  
Performing Organization:  
City and State

Dr. Arthur E. Bogden  
Mason Research Institute  
Worcester, MA

Contract Number: N01-CB-74175

Starting Date: 6/28/79

Expiration Date: 4/27/82

Goal: To function as a service facility for the cryopreservation, storage, and distribution of biologically characterized and monitored (a) animal and human mammary and endocrine related tumors transplantable in vivo, and (b) cell lines of human and animal breast tumor origin established in in vitro culture, for use by the Breast Cancer Task Force and other selected investigators.

Approach: Cryopreservation of mammary and endocrine related tumors of animal and human origin, as well as human breast tumor cell lines: (a) submittal of tumors and cell lines by qualified investigators able to furnish pertinent background information; (b) biological characterization of transplantable tissues, both pre- and post-cryopreservation, by determination of growth curves, specific effects on host organs, host (syngeneic and xenogeneic) survival, serum hormone levels, histology, karyology, response to ablative procedures; (c) cell lines tested for viability, plating efficiency, freedom from contamination and tumorigenicity by nude mouse implantation; (d) characterized tissue and cell lines preserved in Linde liquid nitrogen freezers according to well recognized, proven procedures.

Progress: From January 1, 1981 to December 31, 1981, 141 animal tumors have been requested and shipped to 84 different investigators. Of these, 44% were neoplasms of mammary origin and 9% were anterior pituitary tumors. Thus, 53% of the tumors requested were related in some way to studies on mammary cancer. Some tumors of connective tissue origin were requested to be used for control purposes. Requests were received from investigators in 22 different states and 5 different foreign countries. Of the 62 mammary tumors requested 12 or 19% were for the 13762 rat mammary adenocarcinoma. Currently, there are 221 different animal tumors stored in 5,550 ampules in 7 liquid nitrogen tanks. Two investigators requested the rat -lactalbumin and its antibody and one requested both the rat and human -lactalbumin and the antibodies. Antisera to various types of animal and human collagen and procollagen are cryopreserved and will be available for distribution within a few weeks. Currently there are 31 different cell culture lines in the bank. During this report period 89 shipments were made to 74 different laboratories which is an increase of 40% over the previous year. Twelve shipments were made to foreign countries. There is a greater demand for growing cultures rather than the cryopreserved specimens. Four new parathyroid hormone responsive clones of rat osteogenic sarcoma cell lines were incorporated into the bank. Catalogs of available animal tumor lines and cell culture lines are available for distribution to the scientific community upon request.

Project Officers: Chester V. Piczak  
Program: Breast Cancer Experimental Biology  
FY 82 Funds: \$118,766

# CONTRACT RESEARCH SUMMARY

Title: The Relationship Between Thyroid Disease and Breast Cancer

Principal Investigator:	Dr. Farahe Maloof
Performing Organization:	Massachusetts General Hospital
City and State:	Boston, MA

Contract Number: N01-CB-84230

Starting Date: 9/30/78

Expiration Date: 9/29/82

Goal: To examine the possible association of breast cancer with prior thyroid dysfunction.

Approach: This is a retrospective cohort study designed to measure the rates of breast cancer in women with and without prior thyroid disease. A mortality study will follow 7,000 women with thyroid disease seen at the Thyroid Clinic of the Massachusetts General Hospital between 1925 and 1975, and 7,000 matched population controls; 2,000 women seen at the Clinic but without identified thyroid disease will also be followed. Rates will be compared in women with various types of thyroid disease. A morbidity study will follow 1,600 women with previous thyrotoxicosis; this will include 500 women who became hypothyroid following the treatment and 500 who did not. Rates of breast disease will be compared according to mode of treatment as well as externally compared with rates from the Connecticut Tumor Registry.

Progress: Mortality Study: The card file of patients treated at the Thyroid Clinic was reviewed. From the 45,000 names, 13,400 women satisfied the study criteria, almost twice as many as had been anticipated, allowing us to increase the sample size. This included 3,140 women with no thyroid disease. Pertinent medical data and follow-up information have been abstracted from all available records (12,100), and the information computerized. From intensive followup search on the patients, as of March 31, 1982, death certificates for approximately 5000 patients, have been found and copied, and an additional 350 women located. One control woman was sought from the available Massachusetts Residents' List for each thyroid patient who resided in the state at the time of diagnosis; 10,200 women from the same neighborhood and within two years of age of the case were selected. More than 1000 Residents' List women's death certificates have also been located and abstracted, and the information computerized. Clean-up of data thus far is 85% complete.

Morbidity Study: A computerized roster of the 1,955 women in the USPHS Thyrotoxicosis Follow-Up Study has been compiled; 91% of these have been located--973 have completed questionnaires, 713 have died and copies of death certificates have been obtained, 92 have refused, and efforts are continuing to trace the remaining 177. All information obtained thus far has been entered into the computer, as have the records from the USPHS Thyrotoxicosis Follow-up Study. To date, 500 medical records have been abstracted and preliminary analysis begun. All women who agree to a current clinical evaluation of thyroid function are being examined (590 so far, original estimate 575); 59 re-examinations have also been completed (60 aimed for).

Project Officers: Elizabeth P. Anderson, Ph.D., Bruce Nisula, M.D.  
Program: Breast Cancer Epidemiology  
FY 82 Funds: 0

# CONTRACT RESEARCH SUMMARY

Title: Longitudinal Studies of Biologic Markers in Breast Cancer Patients

Principal Investigator:	Dr. Morton K. Schwartz
Performing Organization:	Memorial Hospital
City and State:	New York, NY

Contract Number: N01-CB-74206

Starting Date: 8/1/77

Expiration Date: 12/31/82

Goal: To assay human breast cancer tissue and sera prior to mastectomy and throughout the clinical course of the disease for potential markers, to determine how effective such markers would be for early detection on breast cancer and its recurrence, and to gain an understanding of the relationship between the concentration of the marker substances in the individual tumor and in the serum levels of the host.

Approach: Serum and tissue specimens from patients entering for diagnosis and for primary therapy are assayed for carcinoembryonic antigen, sialyltransferase, phosphohexose isomerase, spermine, ferritin, lactic dehydrogenase and its isoenzymes,  $\beta$ -human chorionic gonadotropin,  $\gamma$ -glutamyltranspeptidase  $\alpha$ -fetoprotein and IgA. In tissue alone, hormone receptors, total protein, DNA and glucose-6-phosphate dehydrogenase are measured and in serum aspartate aminotransferase, alkaline phosphate, calcium and albumin. Serum specimens are collected prior to surgery, before discharge and then sequentially every 3 months the first year and every 6 months thereafter. The tissue is a portion of that obtained for hormone receptor assay at the time of biopsy or mastectomy. Tissue and serum biochemical data are compared and changes and differences related to the clinical course of the patient as well as demographic and pathology findings.

Progress: Based on an analysis of biochemical substances and clinical variables, mathematical model based on logistic regression analysis has been developed which successfully predicted over 90% of tumor recurrences. This model is being tested.

Project Officer: Donald E. Henson, M.D.  
Program: Breast Cancer Diagnosis  
FY 81 Funds: 0

CONTRACT RESEARCH SUMMARY

Title: Lipid Levels and Cholesterol Metabolism in Relation to Human Breast Cancer Risk

Principal Investigator:  
Performing Organization:  
City and State:

Dr. Angelos E. Papatestas  
Mount Sinai Medical Center  
New York, NY

Contract Number: N01-CB-84318

Starting Date: 9/30/78

Expiration Date: 9/29/82

Goal: To evaluate whether dietary or metabolic factors influence breast cancer risk by inducing systemic and/or local changes at the target organ.

Approach: Case-control study. Subset matched for age, ethnicity, and menopausal status. Data on past medical and family history, obesity, and dietary nutrients. Determinations of serum lipids and fecal steroids. Case-control differences in these variables are evaluated. Associations with known risk factors, the presence of benign breast disease (BBD) or with tumor characteristics (tumor stage, hormone receptors) are examined.

Progress: Accrual of patients was terminated March 31, 1982, and laboratory analyses completed in April, 1982. Cleaning of the data file was completed in May, 1982. Analyses of case-control differences in the several variables under consideration is under way; multivariate analysis is being performed, and both confounding and possible interactions are being examined. Preliminary data suggest that cases have both higher serum lipid levels and fecal steroid excretion compared to controls. Subgroups defined by degree of obesity and by menopausal status have been examined; in lean women fecal steroids appear to be the best differentiating parameter, in obese women free fatty acids and in postmenopausal women serum cholesterol. The influence of dietary habits and bowel habits on fecal steroid excretion is being evaluated. A discriminant analysis is planned to determine if serum lipid values can predict fecal steroid excretion. We are testing a theoretical model of tumor promotion based on the hypothesis that the presence of any of the (metabolic) states that have as a common denominator the potential for increased estrogen production would indicate increased breast cancer risk.

Project Officers: Elizabeth P. Anderson, Ph.D., Kenneth Lippel, Ph.D.

Program: Breast Cancer Epidemiology

FY 82 Funds: 0



# CONTRACT RESEARCH SUMMARY

Title: Interrelationships Among Diet, Steroid Hormone Metabolism, and Human Breast Cancer

Principal Investigator: Dr. Marvin A. Kirschner  
Performing Organization: Newark Beth Israel Medical Center  
City and State: Newark, NJ

Contract Number: N01-CB-84229

Starting Date: 9/30/78

Expiration Date: 3/29/82

Goal: To investigate steroid hormone production rates and metabolism in relation to obesity and dietary patterns.

Approach: This will be an in-depth study of androgen, estrogen and cortisol production, metabolism, and excretion in a cohort of 40 obese women undergoing drastic weight reduction and diet manipulation. The women will be studied prior to weight loss, after weight loss and stabilization on a high protein, low fat diet, and where possible after isocaloric exchange to a more typical high carbohydrate, high fat diet. Values will be compared under three sets of conditions and further compared with those in lean, age-matched controls.

Progress: In the final cohort, baseline studies were carried out on 83 obese, young women (mean weight 224 pounds); despite unexpectedly high drop-out during weight reduction, the women studied following normalization at ideal body weight numbered 15 as of March, 1982, and will probably total 21 before continuing work on this project is eventually completed. In addition, 33 normal, age-matched women have been studied during normal diet, high protein diet and high carbohydrate diet. The data assembled to date suggest that: (1) obese women exhibit several abnormalities of steroid hormone production and metabolism, namely increased production of androstenedione, increased extragonadal metabolism of androstenedione to estrone, increased estrone production rates (via extragonadal pathway), possibly decreased conversion of androstenedione to testosterone, and increased excretion of urinary androgen metabolites androstosterone and etiocholanolone; (2) with weight loss and normalization of body weight there is reversal of androstenedione and estrone production rates toward normal, but metabolic alterations such as clearance rates of androstenedione and extragonadal conversion of androstenedione to estrone remain abnormally high; (3) in normal women, major alterations in dietary components (high protein and high carbohydrate diets) do not result in appreciable changes in these hormone parameters.

Project Officers: Elizabeth P. Anderson, Ph.D., Lynn Loriaux, M.D.  
Program: Breast Cancer Epidemiology  
FY 82 Funds: 0



## CONTRACT RESEARCH SUMMARY

Title: Suppression of Endocrine Function by Systemic Agents for Breast Carcinoma Therapy

Principal Investigator:  
Performing Organization:

Dr. Richard J. Santen  
The Pennsylvania State University  
The Milton S. Hershey Medical Center  
Hershey, PA

City and State:

Contract Number: N01-CB-53841

Starting Date: 5/15/75

Expiration Date: 5/14/83

Goal: To produce suppression of endocrine function with aminoglutethimide (AG), to establish the mechanism of steroid inhibition, and to compare the effects of surgical adrenalectomy, antiestrogen therapy, and aminoglutethimide in human breast carcinoma.

Approach: Female patients with inoperable, recurrent or metastatic breast carcinoma and whose tumors are either estrogen receptor positive or unknown were selected for study. Women were randomized into two separate therapeutic trials: a comparison of medical adrenalectomy (AG + hydrocortisone) vs. surgical adrenalectomy, and of AG vs. the antiestrogen, tamoxifen. Extensive endocrine studies evaluate the effects of AG on extra-adrenal estrogen production, androgen, progesterin, and prolactin secretion, and steroid metabolism.

Progress: Women with metastatic breast cancer were entered into protocols utilizing surgical adx, medical therapy with aminoglutethimide (AG) and tamoxifen (Tam). In a randomized trial of 96 patients, a 53% objective response to AG plus hydrocortisone (HC) and a 43% regression rate to surgical adx were observed. Medical treatment with AG and surgical adx produced similar reductions of estrogen levels, whereas androgen secretion was preserved in the medical group. A randomized trial of AG-HC vs. the antiestrogen, Tam, entered 61 patients. Forty-eight percent responded objectively to AG-HC and 42% to Tam ( $p=NS$ ). Tam-resistant women may respond secondarily to AG-HC. Hormonal levels in 147 AG-treated patients revealed equal estrogen suppression in objective responders as in patients with progressive disease but DHEA-S and androstenedione levels were higher during treatment in patients with progressive disease. Overall, these studies suggest that AG-HC is a logical alternative to surgical adx and produces clinical responses which probably differ from those induced by Tam.

A major question addressed during the last contract year concerned the action of AG directly on hormone synthesis by breast cancer tissue itself via the enzyme aromatase. Studies showed that 21 of 25 human breast cancers contained aromatase activities and that they could be inhibited with AG in a dose dependent fashion. Additional studies in the dog are being carried out to evaluate certain enzyme inductions by AG in the adrenal glands and in the liver. Nineteen publications resulting from this contract have appeared since December 1980 or are in press.

Project Officer: Mary E. Sears, M.D.  
Program: Breast Cancer Treatment  
FY 82 Funds: \$27,000

# CONTRACT RESEARCH SUMMARY

Title: Prognostic Significance in Breast Cancer of Regional Lymph Node

Principal Investigator:	Dr. Susanna Cunningham-Rundles
Performing Organization:	Sloan-Kettering Institute
City and State:	New York, NY

Contract Number: N01-CB-84228

Starting Date: 9/1/78

Expiration Date: 12/31/81

Goal: Evaluation of the relevance of regional lymph node immune response to prognosis in breast cancer. This will require an assessment of cell-mediated immune reactivity in well-standardized in vitro assays to defined antigens associated with the development of disease.

Approach: Immunological studies will be performed on lymph node cells of 100 patients with primary operable breast cancer, using a total of 4-6 lymph nodes per patient including both negative and positive nodes, ideally from each level (I-III). Control lymph nodes will be obtained from 50 other patients. The assays to be done on both peripheral blood and lymph node mononuclear cells include leukocyte migration inhibition factor (LMIF) and lymphocyte transformation tests, the latter comprising PHA, Candida, E. coli, T-antigen and MuMTV. A third class of newer tests includes natural killer cells, suppressor cells, cell surface markers and monocyte function assays. Clinical and demographic data as well as histopathologic data on tumor size and histologic type will be obtained. Three year follow-up information on recurrence and survival status will be recorded. Correlation of all factors will be statistically analyzed.

Progress: The data indicate that women with tumor involved lymph nodes respond more frequently to MuMTV than women who do not have tumor involved nodes. The magnitude of the response in positive responders is similar in both cases and therefore there is no reason to suppose that different antigenic sites are being recognized in the two groups of patients. In both groups there were examples of tumor associated suppression of the immune response. Examination of responses to non-specific mitogens, microbial B Lymphocyte activators and the antigen C. albicans demonstrated that broadly acting suppressive mechanisms were not part of this phenomenon. The data provide a plausible explanation for the general absence of tumor associated suppression that we have reported and allow speculation as to the possible critical significance of local suppression that is specific. Direct correlation with tumor size, lymphocyte sub-populations, and tumor type was not found. The data indicate that both LMIF production and proliferative response provide valuable information related to secondary response. Evidence for gradient of response was found and leads to a suggestion of regulated interaction between peripheral blood compartment and regional lymph nodes.

Project Officer: Donald E. Henson, M.D.  
Program: Breast Cancer Diagnosis  
FY 82 Funds: 0

# CONTRACT RESEARCH SUMMARY

Title: Longitudinal Studies of Biologic Markers in Breast Cancer Patients

Principal Investigator:	Howard H. Sussman, M.D.
Performing Organization:	Stanford University School of Medicine
City and State:	Stanford, CA

Contract Number: N01-CB-74086

Starting Date: 8/1/77

Expiration Date: 7/31/82

Goal: To assay human breast cancer tissue and sera, prior to mastectomy and throughout the clinical course of the disease, for potential markers to determine how effective such markers would be for early detection of breast cancer and its recurrence, and to gain an understanding of the relationship between the concentration of the marker substances in the individual tumor and in the serum levels of the host.

Approach: Tumor and sera of patients presenting initially for diagnosis and primary therapy (Category 1) will be assayed for the fetoplacental proteins, i.e., placental alkaline phosphatase (PAP), human chorionic gonadotropin (hCG)  $\alpha$  and  $\beta$ , and carcinoembryonic antigen (CEA); and tests on sera will be repeated sequentially every three months. Patients developing recurrences (Categories 2 and 3) will be included with a study population from the Northern California Oncology Group. Another objective of the study will be to identify new ectopically synthesized placental membrane proteins, which may be common to both trophoblasts and neoplastic breast tumor cells.

Progress: Serum assays have been continued on the 125 patients participating in the study. One patient from Category 1 has relapsed into Category 2. Three of her four marker levels increased, with two of these becoming abnormal, in the period of three to eight months prior to the diagnosis. The patient has since declined to be followed. A receptor specific for transferrin has been found in neoplastic breast tumors. Microsomes from these tumors demonstrate 11-35% binding of transferrin, while those from normal breast tissue show only 2-3% binding. A radioimmunoassay has been set up to detect the level of transferrin receptor on cell membranes. Preliminary results, expressed as g receptor/mg protein, show normal breast (0.2) and a benign male breast (0.4) to have low levels, while placenta (33.6) has a high level. Eleven breast cancer tumors have been assayed and those levels fall between these two limits. Data on the 400 subjects from whom we have at least one blood sample, including controls, in continuing to be collected and entered into a computer for future evaluation.

Project Officer: Donald E. Henson, M.D.  
Program: Breast Cancer Diagnosis  
FY 82 Funds: 0

## CONTRACT RESEARCH SUMMARY

Title: Epidemiology of Benign Breast Disease

Principal Investigator:	Dr. Gary H. Spivey
Performing Organization:	University of California
City and State:	Los Angeles, CA

Contract Number: N01-CB-74202

Starting Date: 8/15/77

Expiration Date: 1/14/82

Goal: To examine the epidemiology of major types of benign breast disease and to compare identifiable risk factors for benign diseases with known risk factors for breast cancer. To examine a histologic classification scheme based on grading of cellular atypia in comparison to the conventional histologic classification.

Approach: Women with first biopsies for benign breast disease in participating hospitals are identified from pathology records and selected to maximize representation of various histologic subtypes. Biopsy slides of participants are reviewed by two pathologists and classified according to two classification systems. A subcategory of women with breast cancer is included. Two types of controls are used, a hospital control and a friend control. Epidemiologic information is obtained by a questionnaire covering a wide range of topics including environmental influences; familial cancer patterns; medical, maturation, and reproductive history; and other endocrine-related subjects. Different histologic types of benign breast disease are compared to each other and to breast cancer.

Progress: The final data set of interviewees includes (1) 877 benign breast disease cases and 445 of each type of control (827 matched pairs, 406 with hospital controls and 421 with friend controls); and (2) 214 breast cancer cases matched with 189 hospital controls and 191 friend controls (163 matched triplets - cancer case, matched hospital control, matched friend control). This is one of the largest studies to date on benign breast disease. Univariate analyses have been carried out on all variables, but not yet adjusted for possible confounders. Logistic regression analyses have also been carried out to explore the joint effects of a number of variables on the risk of disease; more of these are still under way. In several instances, the benign breast disease cases differed from controls in the same direction as breast cancer cases, but to a lesser degree, consistent with the hypothesis that a subset of benign breast diseases are at increased risk of breast cancer and would show a similar pattern of risk factors (e.g. taller and thinner at menarche). Also consistent with this is the fact that the breast cancer cases had more history of benign breast disease than did the controls, and that the cancer cases had more family history of both cancer and benign breast disease. The analyses are also exploring risk factors for histopathologic subcategories of benign breast disease. Differences were observed with the two different types of controls; data on such effects are very scarce, and careful analysis of this can be valuable for epidemiologic methodology. Several publications can be anticipated; one has already been accepted for publication, another written, and at least three more planned for the near future.

Project Officers: Elizabeth P. Anderson, Ph.D., B.J. Stone, Ph.D.

Program: Breast Cancer Epidemiology

FY 82 Funds: 0



# CONTRACT RESEARCH SUMMARY

Title: Biologic Characterization of "Premalignant" Human Mammary Epithelial Hyperplasias

Principal Investigator:  
Performing Organization:  
City and Date:

Dr. Hanne M. Jensen  
University of California  
Davis, CA

Contract Number: N01-CB-84316  
Starting Date: 9/18/78

Expiration Date: 12/31/81

Goal: To establish if angiogenesis is a reliable marker for precancer conditions of the human breast and to characterize by biochemical and immunological methods the type(s) of lesions that possess angiogenic capacity from those that do not.

Approach: Fresh, parenchymal structures and hyperplastic lesions stained with methylene blue chloride are isolated and studied for angiogenic potential by transplantation onto the iris of female rabbits. 6 $\mu$  thick frozen sections of parts of isolated structures and lesions are stained with fluorescein labeled anti-human immunoglobulins G, A and M, to characterize them immunologically and with fluorescein labeled estrogen and progesterone to detect hormone receptors.

Progress: Results from 673 transplants assayed for angiogenesis continue to show that histologically benign lobules from cancer associated breasts are more likely to stimulate neovascularization than lobules removed from non-cancerous breast. Normal lobules derived from cancer associated breasts were significantly more often angiogenic than those from non-cancer associated breasts. These data continue to support the hypothesis that some angiogenic factor is present in low concentration in many normal structures, but that a substantial increase in this factor is associated with malignant transformation and may precede the morphologic manifestation of cancer.

Data further suggest that the angiogenic factor is not limited to the lobules, but also occurs in cysts within the breast and in secretions. The angiogenic factor is found in cyst fluid from both cancer associated and non-cancerous associated breasts.

Project Officer: Donald E. Henson, M.D.  
Program: Breast Cancer Diagnosis  
FY 82 Funds: 0



# CONTRACT RESEARCH SUMMARY

Title: Prediction of Hormone Dependency in Human Breast Cancer

Principal Investigator:  
Performing Organization:  
City and State:

Dr. Elwood V. Jensen  
University of Chicago  
Chicago, IL

Contract Number: N01-CB-14358  
Starting Date: 6/16/66

Expiration Date: 7/15/83

Goal: To develop and improve techniques that will increase the predictability of response of human breast cancers to endocrine therapy.

Approach: Since 1966, Dr. Jensen has been studying estrogen receptors (ER) in human breast cancer tissues and the relationship between ER content and response to endocrine manipulative treatments. Now that the predictive value of ER measurements has been established, attention is being directed toward development of simple, inexpensive assay procedures for ER in breast cancers and evaluation of the ability of an ER assay on the primary tumor, carried out at the time of mastectomy, to predict subsequent response to endocrine therapy.

Progress: During the concluding stages of this contract, a major effort has been devoted to utilizing various monoclonal lines of antibodies to human estrophilin to provide dependable and inexpensive immunochemical and immunocytochemical methods for the analysis of breast cancer specimens. So far we have obtained eleven different monoclonal antibody preparations, each of which recognizes a different antigenic determinant on the human estrophilin molecule. A combination of two of these antibody preparations can be used in a sandwich technique in which one antibody, fixed to a polystyrene bead, serves to absorb the receptor from the tumor cytosol and the second antibody, labeled either with radioactive iodine or with a chromogenic enzyme, serves as a marker for the bound receptor. A suitable combination of antibodies has been found which provides a simple immunoradiometric procedure that yield results with human breast cancers that agree well with those obtained with the currently employed technique of sucrose gradient centrifugation. This analytical system (using the enzyme-linked colorimetric marker rather than the radioactive one) is now being evaluated in four independent laboratories. Our antibodies also detect and localize estrogen receptor in breast cancer sections by an immunoperoxidase technique. When perfected this should provide a simple and useful means of identifying the presence of receptors within individual cancer cells. Also the immunocytochemical procedure makes it possible to characterize, retrospectively, primary tumors of patients who previously have had mastectomy without receptor analysis and who now have advanced disease with no metastases available for assay. Retrospective examination continues of the records of more than 1000 patients whose breast cancers were analyzed for estrogen receptor content in our laboratory between the years 1966 and 1976, with the objective of obtaining as much additional information as possible concerning various parameters relevant to the course of their disease and response to endocrine therapy.

Project Officer: Mary E. Sears, M.D.; Ihor J. Masnyk, Ph.D.  
Program: Breast Cancer Treatment  
Fy 82 Funds: \$10,000

## CONTRACT RESEARCH SUMMARY

Title: Detection of Immune Complexes in Sera of Patients with Breast Cancer

Principal Investigator:	Dr. M. Edward Medof
Performing Organization:	The University of Chicago
City and State:	Chicago, IL

Contract Number: N01-CB-84224

Starting Date: 9/1/78

Expiration Date: 12/31/81

Goal: To assay sera from patients with breast cancer, benign breast disease, and normal volunteers for immune complexes (ICs) employing multiple techniques for IC quantitation, then to correlate levels with the presence or absence of tumor, or benign disease, tumor progression, response to therapeutic intervention and clinical course; to determine how hormonal status, past history and other clinical factors influence results, and to establish the relationship between IC data, pathologic features or other laboratory parameters.

Approach: Four methods for the detection of ICs (Raji cell assay, conglutinin-binding assay, solid phase Clq assay, and Clq-PEG precipitation assay) were established in this laboratory and run on sera from new patients with breast cancer (20-30) or with benign breast diseases (40-50). Serial samples from patients receiving and not receiving chemotherapy and/or Tamoxifen following mastectomy were also studied. Efforts were made to obtain sera from other investigators in the breast cancer study group, assay these sera, and correlate IC data with other markers. Serum samples are assayed for soluble and insoluble, complement bearing and IgG-Fc accessible ICs. Data will be correlated with type of mastectomy, stage of cancer, histologic type of tumor, including estrogen receptor status, histology of regional lymph nodes, as well as presence of CEA and K-casein,  $\beta$ -human chorionic gonadotropin and prolactin levels in selected cases.

Progress: An analysis of immune complex levels in 434 serum samples obtained from 332 patients with breast disease by four independent assay methods indicates that the Raji cell radioassay is the most effective in differentiating patients according to the presence or absence of non-cancerous breast disease. There seems to be a trend toward an association between elevated levels of immune complexes and the presence of fibrocystic disease. There also seems to be a correlation between immune complex levels as determined by the Raji assay and the estrogen receptor content of tumors. Immune complex results from all four assays correlate with the stage of the cancer, but do not correlate with the histologic type. Levels in patients with stage II cancer are higher than those in patients with stage I cancer by all four assays. Levels in patients with lobular cancer are higher than those with ductal cancer by the Raji cell assays while the reverse is true by the conglutinin assay.

Project Officer: Donald E. Henson, M.D.  
Program: Breast Cancer Diagnosis  
FY 82 Funds: 0

# CONTRACT RESEARCH SUMMARY

Title: Benign and Non-Invasive Breast Lesions in Groups at Different Risk for Breast Cancer

Principal Investigator: Dr. Sue Amelia Bartow  
Performing Organization: University of New Mexico  
City and State: Albuquerque, NM

Contract Number: N01-CB-84231

Starting Date: 9/30/78

Expiration Date: 9/29/82

Goal: To investigate whether population groups with low incidence of breast cancer also have a low prevalence at autopsy of benign breast lesions.

Approach: Combining the resources of the population-based New Mexico Tumor Registry, the State's Medical Investigator's Office and the Medical School Departments of Pathology and Radiology, we propose to examine and compare benign breast lesions in three ethnic groups in an autopsy series--Anglos, Spanish Americans, and American Indians. These three groups are at differing risk for breast cancer. The autopsy series to be examined will include approximately 500 women: 230 Anglos, 140 Spanish American, and 130 American Indian. Breast lesions will be classified pathologically. Radiological studies of the specimens by both clinical mammography and high resolution techniques will be compared with histopathological findings. Medical, reproductive, and related history of each case will be obtained from the family, or medical records if at all possible.

Progress: As of June 12, 1982, 426 cases have been accessioned into the study (Anglo 229, Spanish American 103, and American Indian 94). Autopsies are now being preferentially performed on the low risk Spanish and Indian women. Of the cases accessioned, 414 have been examined grossly by the pathologist, and full histology has been read on 320 by one pathologist and on 280 by the other. Evaluation of the radiographic studies has been performed on 330 cases. Medical history information is being pursued vigorously. Some of the family contact letters and questionnaires have been simplified to bring increased responses. Additional critical information on Indian women is being obtained through review of existing Public Health Service records, e.g., on parity and age at first childbirth. The data collected to date from pathology and medical history have been completely entered into computer storage; we are in process of entering the data from the radiographic evaluation. Review of the data from 132 interviews completed indicates that much of the critical history is reliable and relatively complete. Interesting differences are appearing in breast histology of low risk vs. high risk women. The implications are that the breasts of low risk women undergo more complete involution of the non-fat breast tissue and hence a change to predominantly fatty pattern earlier in life, whereas in high risk women more abundant lobular tissue persists with advancing age; this could give a high risk woman more tissue at risk for carcinogenesis. One publication on this has already appeared.

Project Officers: Elizabeth P. Anderson, Ph.D., Louise Brinton, Ph.D.  
Program: Breast Cancer Epidemiology  
FY 82 Funds: \$28,000

## CONTRACT RESEARCH SUMMARY

Title: Methods to Predict Chemotherapy Sensitivity

Principal Investigator:	Dr. Russell Hilf
Performing Organization:	University of Rochester
City and State:	Rochester, NY

Contract Number: N01-CB-74204

Starting Date: 7/15/77

Expiration Date: 7/14/82

Goal: To develop a reliable prognostic test that will predict the efficacy of chemotherapeutic agents in the treatment of individual breast cancer patients.

Approach: This project has performed a battery of selected enzyme assays in primary and metastatic breast cancer specimens and has developed mathematical models based on the biochemical profile. The enzymes include lactate dehydrogenase, pyruvate kinase, glucosephosphate isomerase, isocitrate dehydrogenase, phosphoglucomutase and glucose 6-phosphate dehydrogenase; DNA and protein levels will be measured. Hormone receptor assays and histopathology examinations have also been performed. Data concerning the patients' biological and oncological histories are being acquired, evaluated, and stored. Analyses of the combined data will test the predictive accuracy of the mathematical model constructed from the biochemical profile.

Progress: A logistic regression model, utilizing the activities of certain selected glycolytic enzymes and ER status measured on primary or recurrent lesions, has been applied to predict for response to combination chemotherapy regimens administered to women with advanced breast cancer. The clinical outcome of response or no response was evaluated retrospectively using criteria employed by cooperative group protocols. In 93 cases, 58 of 61 patients classified as non-responders and 22 of 32 patients demonstrating objective responses would have been correctly designated, based on the 50% estimated probability as the level for separation of responders from non-responders. The overall predictive accuracy of this model was 86%, with apparently greater accuracy for prediction of lack of response. Addition of estrogen receptor status to the model imparted no gain in accuracy of prediction. Application of this model to a prospective study is warranted. The manuscript reporting these data has been accepted for publication and will appear in the journal Cancer.

Project Officer: Mary E. Sears, M.D.  
Program: Breast Cancer Treatment  
FY 82 Funds: 0



# CONTRACT RESEARCH SUMMARY

Title: Biochemical Mechanism of Endocrine-Induced Breast Cancer Regression

Principal Investigator:

Dr. William L. McGuire

Performing Organization:

University of Texas Health Science Center

City and Date:

San Antonio, TX

Contract Number: N01-CB-23862

Starting Date: 6/1/72

Expiration Date: 5/31/82

Goal: To obtain new information of the hormonal control of breast tumor growth and to identify those breast cancer patients whose disease will respond to hormonal manipulation.

Approach: To provide correlations of estrogen and progesterone receptor measurements with prognosis at the time of mastectomy and response to endocrine therapies. To evaluate histochemical methods of measuring steroid receptors.

Progress: The contract which ended in May, 1982 accomplished a number of objectives. 1) Estrogen receptor (ER) content correlation with primary breast cancer histological features, 2) correlation of ER and progesterone receptor (PgR) content in predicting recurrence in Stage II breast cancer, 3) analysis of multiple ER assays which revealed a change in status of 24%, 4) affinity purification of ER from MCF-7 human breast cancer cells grown in roller bottles, 5) immunization of male Lewis rats which yielded titers suitable for fusion of the animals spleen cells with a mouse myeloma cell line and, 6) positive ER antibody production by hybridoma lines in culture. To strengthen the stability of the hybrid lines other combinations are under study, especially fusing the immune rat splenic lymphocytes with a rat myeloma line. One hundred and eleven publications have resulted from this project during the existence of the contract.

Project Officer: Mary E. Sears, M.D.

Program: Breast Cancer Treatment

FY 82 Funds: 0





## CANCER DIAGNOSIS RESEARCH PROGRAM

### DESCRIPTION

The Cancer Diagnosis Research Program emphasizes research in early detection, diagnosis (which includes staging and prognosis), tumor localization, and monitoring the changes during therapy or progression of disease. The program also seeks to apply the knowledge obtained to appropriate populations for clinical evaluation. Projects in these areas are frequently concerned with improvement of existing techniques as well as the development of new tests and procedures. Many of the projects in the Cancer Diagnosis Research Program have begun in a more basic area such as, Tumor Biology, Tumor Immunology, or General Medical Sciences for instrument development. As a basic concept becomes potentially useful in one of the areas of cancer diagnosis the project may be transferred to the Cancer Diagnosis Research Program. In the earliest stages, diagnostic research is not necessarily site (or even disease) oriented.

The major objective of the Program is to recognize or detect cancer at the earliest possible stage to allow appropriate therapy to begin. Early detection and early treatment should improve the chances for the control of cancer, decrease mortality from the disease and increase survival and quality of life of those with cancer. Additionally, early detection is providing greater understanding of the natural history of different types of cancer in the early stages of disease.

Since the techniques of cancer detection and diagnosis are useful for many, or all, types of cancer the divisions of the program are not by cancer type or organ site, but by technical discipline. The Diagnosis Research Program consists of projects in five disciplinary categories: Biochemistry, Immunodiagnosis, Cytology, Pathology, and those projects that are clearly Multiple Disciplinary. Research in diagnostic imaging (radiologic and non-radiologic) was transferred to the new Radiation Research Program, DCT.

The distribution of contracts and grants currently included (FY1982) in the Diagnosis Research Program in the various categories is summarized in the following table. Each category is discussed in the sections following the table.

TABLE 1

## CANCER DIAGNOSIS RESEARCH PROGRAM

ALL PROJECT EFFECTIVE DURING FISCAL YEAR 1982

Number	Category	Grants		Contracts	
		Number	Current Funding (in Thousands)	Number	Current Funding (in Thousands)
1	Biochemistry	19	1,512	0	0
2	Immunodiagnosis	28	3,266	13	758
3	Cytology	27	3,431	4	0
4	Pathology	5	398	0	0
5	Multiple Disciplines	1	65	5	2,760
Totals		80	8,672	22	3,518

## CANCER DIAGNOSIS RESEARCH PROGRAM

### 1. BIOCHEMISTRY

Biochemical methods to improve diagnosis and detection of cancer involve the study of a variety of substances such as hormones, enzymes, proteins and metabolic products in the circulation and in other biological fluids, as well as the study of surface characteristics of tumor cells and chemical characterization of tumor cells.

Under hormonal studies, the measurement and mode of action of the multiple forms of human growth hormone are being studied under grant CA-33615. A new grant CA-30687, is designed to study and develop an assay for a group of progesterone-dependent proteins synthesized by the human endometrium to probe their usefulness as discriminants in diagnosis, prognosis and in monitoring response to treatment. Identification, isolation and characterization of thyroid hyperfunction in patients with trophoblastic tumors, especially hydatidiform mole, are being pursued under grant CA-31218. A series of experiments have been proposed in grant CA-29062 to evaluate the clinical use of a material which is cross-reactive to vasoactive intestinal peptide (VIP) as a biochemical marker to refine the diagnosis of acute and chronic leukemias.

A variety of enzymes are being studied for their correlation with different cancers. Creatine kinase isozyme BB and carcinoembryonic antigen (CEA) are being simultaneously measured in sera and malignant effusions from various cancer types to assess their predictive value in diagnosis and in monitoring of disease response during chemotherapy under grant CA-32585. The biochemistry of isozyme 5 of acid phosphatase and its clinical implications in the diagnosis and prognosis of prostatic cancer and of hairy-cell leukemia are topics of investigation for grant CA-31187. Studies of 5'-nucleotide phosphodiesterase isozymes which have been shown to be elevated in patients with known hepatic metastases from mammary carcinoma and melanoma will be continued under grant CA-25376. A basis for characterizing and classifying human lung tumors and revealing essential biochemical pathways vulnerable to chemotherapy by means of quantitative enzyme profiles is being sought under grant CA-25016. Serum, urine and CSF ribonucleases in tissues and body fluids of healthy subjects and breast cancer patients are being isolated, chemically and physically characterized and correlated with disease status under grant CA-19606. In grant CA-25548 human tumor cell lines were characterized by isozyme phenotyping to confirm whether they are bona fide representatives of the tumors from which they were derived. Differentiation of lymphocytes by the enzyme marker, terminal deoxynucleotidyl transferase, (TdT) is being investigated under grant CA-22599 by determination of the relationship of presence or absence of the enzyme to particular cell sets and to tumors representing those cell sets. The differentiative potential of cells transformed by Abelson Leukemia Virus and chemical carcinogens will be further determined.

Proteins as discriminants of cancer are being investigated under several grants. Computer assisted protein analysis of pancreatic secretions is being probed for diagnostic potential in pancreatitis and pancreatic cancer under grant CA-14380. Intercellular matrix proteins are being extracted from human chondrosarcomas in grant CA-23945 and compared with normal articular cartilage to evaluate degree of malignancy and possibly grade chondrosarcomas biochemically as well as morphologically. A new investigator research grant, CA-30667, aims to exploit the differences in lectin binding characteristic of the mucin found

in normal colon and abnormal colon, malignant and premalignant, to predict the risk of developing colon cancer in high risk patients. In studies proposed under a new grant, CA-30627, a factor which is released from specific tumor cells in vivo to the circulation and in vitro to culture medium will be identified and characterized and may form the basis for a general test for the presence of transformed cells.

Urinary nucleoside breakdown products of tRNA are being measured in cancer patients in grant CA-25210 to explore the molecular mechanism responsible for increased excretion in cancer patients. In grant CA-14185 modified purines, pyrimidines, imidazoles and their nucleosides derived from the turnover of human RNA and from the nucleic acid anabolic processes and present in urines of cancer patients will be evaluated as possible quantitative tumor markers.

A "myasthenic" substance found in small cell lung cancer tissue is being studied as a possible aid in detection of lung cancer under grant CA-22885. Gas chromatography of the colonic microbial metabolites in breath will be performed in grant CA-29056 to test the hypothesis that these metabolites may be useful markers for increased risk of developing colonic cancer.

## 2. IMMUNODIAGNOSIS

The portion of the Diagnosis Program classified as Immunodiagnosis can be subdivided into projects dealing with circulating tumor antigens or markers, such as oncofetal antigens, hormones, enzymes, and glycoproteins; projects dealing with tumor associated antigens, research in localization of tumors by radioimmunodetection; studies of lymphocytes in host-tumor relationships, and projects dealing with antibodies to tumors (including immune complexes).

The use of monoclonal antibodies produced by hybridomas is an exciting new development in the area of immunodiagnosis. Much of the work that has been started with xenogeneic antiserum produced in a variety of experimental animals is now being repeated with monoclonal antibody which has the distinct advantage of being specific for a single antigenic epitope. This will lead to a new look at previously known tumor markers of all kinds and a renewed effort toward the identification of new tumor associated antigens.

The carcinoembryonic antigen is the most widely utilized circulating oncofetal tumor marker. There is an ongoing study on the heterogeneity of CEA under grant CA-24376 which seeks to identify and isolate antigenic variants of CEA of the human digestive system. Comparison of new gastrointestinal cancer antigens with CEA and improvement of their clinical use is being undertaken in program project grant CA-04486.

Several projects are investigating the relationship between hormone levels and cancer: two studies of serum calcitonin as a screen for family members of patients with medullary thyroid cancer are supported by grant CA-22595 and contract CB-63994. The role of serum thyroglobulin levels as markers of tumor recurrence in thyroid cancer is being investigated under grant CA-25338. Tumor production of vasopressin, oxytocin, vasotocin and their associated neurophysins will be measured in grant CA-19613 to assess their value in the diagnosis and monitoring of therapy in patients with small cell carcinoma of the lung.



Two studies of enzymes as markers for cancer deal with measurement of serum levels of tyrosinase in patients with melanoma in grant CA-25381 and of UDP-galactosyltransferase in patients with ovarian and breast carcinoma in contract CB-84260. The objective of grant CA-29255 is to develop an immunohistochemical technique for the visualization of a monocyte esterase marker to be employed in the differential diagnosis of subtypes of leukemias. The goal of grant CA-25088 is to ascertain by immunochemical means if specific types of cancer can in part be detected by measurement of RNases in urine and sera which are presumably released by malignant tissues.

Proteins or their degradation products have been detected in body fluids and have demonstrated correlation with the presence of cancer. The role of SAA, a major protein constituent of secondary amyloid, is being investigated as a marker of tumor recurrence, response to therapy and immune function in cancers of the lung and gastrointestinal tract on grant CA-22141. Gp 48, a major group of glycoproteins synthesized and released into organ culture from breast adenocarcinoma specimens, are being assessed for their diagnostic significance in grant CA-24645. A glycoprotein, EDCl, is being monitored in both urine and plasma of breast cancer patients and controls for its immunodiagnostic value under contract CB-84308. Studies are being continued under grant CA-31762 to biochemically characterize a glycoprotein surface antigen found on myeloblasts of leukemia patients, prepare antiserum to it and monitor its efficacy in predicting relapse of leukemia patients in remission.

Tumor associated antigens studies are under way for many organ sites: urogenital tract, CA-27213; melanoma, CA-30019; mesothelioma, CA-27081; and colon, CA-26246. The approach in a new grant, CA-31701, will be to determine whether indirect immunofluorescence and immunoperoxidase procedures with monoclonal antibodies developed against human melanoma antigens can localize tumor associated antigens in cryostat and paraffin sections of tumor specimens. The diagnostic and prognostic value of monoclonal antibodies that bind to human prostate adenocarcinoma membrane antigens will be studied under grant CA-27623. Studies of nuclear antigens associated with normal and leukemic human blood cells to determine their usefulness as markers in hematopoiesis are being supported by grant CA-26948. The overall objective of grant CA-32245 is to define and characterize human sarcoma-related mesenchymal antigens with monoclonal antibodies and to explain their association with malignancy.

Several studies are concerned with techniques for labeling antibodies with radionuclides for tumor detection and localization. Grant CA-30255 is directed towards localization of germ-cell tumors and hepatomas which produce alphafetoprotein by labeling of monoclonal antibodies with <sup>131</sup>Iodine, <sup>99</sup>Technetium and <sup>111</sup>Indium. An approach is being tested under grant CA-28462 to develop a better technique for labeling antibodies to human serum albumin (HSA) with <sup>111</sup>In or <sup>99</sup>Tc or <sup>128</sup>I, and studying the effect of various conditions, of conjugation on accelerated blood clearance and retention of immunological activity. The objective of grant CA-17742 is to study conditions favoring the localization of CEA-containing human tumors in animal systems by means of radiolabeled anti-CEA immunoglobulins and total-body photoscanning. Grant CA-25584 is a continuation of the program of CEA-tumor radioimmunodetection and involves planning a clinical trial of patients with proven malignancy to further evaluate its use in initial tumor diagnosis and in the management and clinical staging of cancer patients. Grant CA-29639 will concern itself with the development of a method for melanoma localization using radiolabeled monoclonal antibody fragments against p97 antigen

and imaging by emission tomography. Studies proposed under grant CA-31236 will evaluate the role and accuracy of radiolabeled anti-AFP and anti-hCG in detecting testicular cancer using total-body scintigraphy.

Lymphocytes and monocytes in host tumor responses are the subject of several projects. The development of clinically useful diagnostic tests based on the selective binding of bacteria and antibody-coated bacteria to lymphocyte subpopulations is being undertaken under grant CA-29552 and CA-21399.

Circulating antibodies to tumor antigens are being studied in patients with melanoma, in contract CB-74120. Investigations of the expression of the major virion glycoprotein gp52 of MMTV on late-occurring mammary tumors in mice and its potential as a tumor marker are being pursued under grant CA-28305.

In addition to the research grants and contracts, there are contracts for the collection, storage and distribution of serum samples from patients with cancer and other acute and chronic diseases and during the course of therapy for certain cancers. These contracts, CB-04350, CB-74213, CB-74138, CB-84258 and CB-84296, have been invaluable for the rapid evaluation of a wide variety of serologic tests for cancer that come to the attention of NCI. Two contracts, CB-14359 and CB-14339 support the statistical services which are required for the Serum Bank programs. CEA levels on blood stored at the NCI-Mayo Serum Bank are performed under contract CB-23854. A tissue culture bank for cell lines which can be utilized by an investigator for research in cancer immunodiagnosis is maintained under contract CB-14351.

### 3. CYTOLOGY

Diagnostic cytology research projects include the development of automated instrumentation and cell markers that can be used to differentiate normal and atypical cells. Instruments undergoing development and testing are those of high resolution slide based: grants CA-13271, CA-28833, CA-27313, CA-31049, and CA-31718, and flow fluorometric types: contract CB-33862 and grants CA-30582 and CA-33148. Under grant CA-27313, new automated morphometric image analysis techniques are being developed to establish criteria for the identification and classification of cells in sputum; and under grants CA-28833 and CA-32345, image-processing techniques are being developed and applied to rapidly screen cells from urinary bladder and voided human urine respectively. Classification of gynecologic cells based on computer analysis of their digitized images is being supported under grant CA-13271; while a clinical trial using a high resolution image analysis system to analyze monolayers of Papanicolaou stained cells for detection of cervical cancer is supported under grant CA-31049.

Development, construction and testing of an ultrafast optical scanner for microscopic specimens, grant CA-24466, will make possible the automated search for abnormal, transformed or cytochemically marked cells in microscopic preparations. A prototype scanner is now under fabrication.

Single cell classification algorithms employing the current state of the art in digital image processing, scene segmentation and image acquisition hardware are developed and tested in contract CB70314. A cost/utility analysis indicates that the chosen algorithms can operate with the performance sufficient to support cost-effective automated screening for cervical cancer. The continuation of this work is under the support of grant CA-31718. An automated screening system has

been designed and is under fabrication. The system utilizes digital image analysis approach to analyze epithelial cells.

The testing of a multidimensional slit-scan instrument which uses a combined static and flow system is continued. A spectrum of normal and abnormal gynecologic specimens are used. The study will determine the system characteristics including rate and causes of false alarms, contract CB-33862. The instrument is sensitive to the entire spectrum of abnormality of the cells tested. A new system of less complexity is being developed under grant CA-30582. This system will provide the same multidimensional slit-scan information as the first instrument but substantially more rapidly and more accurately. The same technology is being applied to analyze exfoliated cells from the bladder epithelium to detect cancer of the urinary bladder, grant CA-33148.

Two different systems are under development to place individual sorted cells on slides for subsequent retrieval and analysis, grants CA-28886 and CA-28706. The systems will make possible the association of any cell sorted and placed on a microscope slide with its flow microfluorometric data; in addition, a fluid cell sorter is being designed under grant CA-32314. This cell sorter will be used in conjunction with a multidimensional slit-scan flow system.

Chemical synthesis of cytochemical probes, grants CA-28770 and CA-30148, with sharp fluorescence emission spectra will enhance multiple staining of cells for flow systems and for cells on slides in static systems. Cellular fluorescent markers under investigation also include acridine orange, contract CB-33862; and grants CA-30582 and CA-33148. Cell surface antigens may also be used as markers for abnormal cells. With appropriate fluorescent tagging of these antigens, normal and abnormal cells may be differentiated by automated instruments. Potentially useful markers being studied are Herpes simplex virus related antigens, grant CA-28724. Also analysis of DNA, RNA, protein, nucleolar antigen and cell volume are explored under grant CA-28771 to identify atypical cells in clinical material. One HSV marker protein has been shown to be HSV-2 specific and is being investigated for its use in uterine cervical cell labeling. In addition, cytochemical and biophysical probes of nuclear and cytoplasmic structure are being used to distinguish normal and malignant cells and to discriminate between cycling and noncycling cells, grant CA-28704.

Analysis of the performance of automated systems, comparison of instrumental or system classification and manual microscopic classification utilizing standard morphologic criteria is studied under contract CB-74190. The classification algorithms developed under this contract have been found to yield results comparable to human observers provided that good manual segmentations are used.

Flow cytometry is used in grant CA-27283 to develop an assay for early detection of transformed cells (by chemical carcinogens) differentially labeled with fluorescamine. Hyaluronic acid (HA) synthetase activity is found to be stimulated significantly during the early stages of transformation. The regulation of HA synthesis by transformed cells is being investigated and characterized.

To better understand and treat lymphoproliferative disease, flow microfluorometric analysis of human lymphoid malignancies is under investigation, grant CA-23393. Malignant cell subpopulations are identified using correlated analysis of membrane antigens, DNA and light scatter signals. In addition, it is hoped that the ploidy and cell cycle kinetic parameter of these cells will aid



in the diagnosis, classification, scheduling and monitoring of treatment. A combined flow cytometric cell-sorting autoradiographic technique is continued in grant CA-25348 to monitor acute leukemias of adults and children and to predict relapse and evaluate treatment protocol. Using fluorescence microscopy and flow microfluorimetry in grant CA-27123, merocyanine 540 is employed to study leukemia to characterize the dynamic changes in the staining pattern during the clinical course and to isolate and characterize hematopoietic progenitor cells. The selective staining of leukemic cells might be a useful adjunct to existing methods of monitoring the course of leukemia and of prognosis of relapse or remission. The procedure may also provide information concerning the origins of leukemic disease. Merocyanine 540 is also utilized to elucidate the relationship between dye binding and the transformed state of cells in grant CA-28921. The potential use of MC540 in clinical medicine is also being investigated. Leukemic cells are also being studied to determine and to test chemotherapeutic agents for their ability to alter patterns of cell differentiation *in vivo*, grant CA-22942. Study of human leukemic and preleukemic blood and bone marrow using the soft agar culture techniques has continued, grant CA-17353, to characterize the status and prognosis of hematoproliferative disorders. Measurements of the quality and quantity of colony formation appear to provide useful indicators of disease status and progression as well as diagnosis and predictions concerning response to therapy of acute nonlymphocytic leukemias and preleukemic states.

In an effort to better understand the gynecologic neoplastic lesions, a quantitative analysis of nuclear DNA content using Feulgen microspectrophotometry is under way, grant CA-24932. Determination of the nuclear DNA changes may reveal the correlation of the changes with regression, persistence and progression of squamous cancer of the uterine cervix. By determining the ploidy patterns, it is possible to differentiate neoplastic from non-neoplastic changes. Earlier findings of carcinoma-in-situ of lung, intestine, ovary and bladder might be possible by using an immunocytologic technique being evaluated in grant CA-26863, to detect carcinoembryonic antigen on exfoliated cells. The data thus far indicate that cytologic examination with anti-CEA staining procedure may increase the accuracy of screening body fluids for malignant cells.

An extensive study to test the feasibility of screening for endometrial cancers in asymptomatic women by means of uterine sampling is supported under contract CB-84233. The data so far indicate that it is possible to detect occult endometrial carcinoma in older (45 years old and over) asymptomatic women by cytology of uterine sampling.

The Ninth Conference on Analytical Cytology was partially supported by grant CA-32959.

#### 4. PATHOLOGY

A new classification of human pituitary adenomas based on electron microscopy and immunocytochemistry has been developed on grant CA-21905. To expand understanding of tumor behavior in the pyriform sinus and the oral cavity, whole organ sections of patients who undergo surgical excision have been studied, grant CA-22101, using microscopic appearance to determine size and extent of tumor, relation of tumor spread to the laryngeal framework and specific routes of tumor spread. Malignant lymphomas and leukemias are being studied on grant CA-26422 by a combination of methodologies including immunologic surface markers,

monoclonal antibodies, cytogenetics, electron microscopy and flow microfluorometry to determine the most valuable tests to provide earlier detection and more precise classification. Using pathological specimens, various tumor markers including estrogen and progesterone receptors, CEA, HCG, AFP, are being evaluated in grant CA-29211.

## 5. MULTIPLE DISCIPLINES

A number of contracts and one grant involve several disciplines and therefore are presented in this last category of multiple disciplines. Grant CA-25582 is studying fluorescence bronchoscopy and photoradiation therapy with prophyrin derivatives.

A collaborative study to determine the potential of chest x-ray imaging and cytological examination of sputum in detection of early lung cancer is being conducted under contracts CB-45007, CB-45037, and CB-53886. A fourth related contract, CB-43868, is concerned with the data management of the collaborative lung study. The study, which was begun in 1974, involves 30,000 men. The screening phase is being concluded, to be followed by a follow-up phase.

A large-scale longitudinal colon cancer screening study using the Hemoccult test for human blood in the feces is the task of contract CB-53862. Several more years of follow-up on the 45,000 subjects will be required to define the usefulness of the screening procedure in detecting early colon cancer.

## Publications

McIntire, K.R.: Non-hormone Markers of Human Lung Cancer. In Colnaghi, M.I., Buraggi, G.L. and Ghione, M. (Eds.): Markers for Diagnosis and Monitoring of Human Cancer, Sero Symposium No. 46. London and New York, Academic Press, 1982, pp. 95-109.

McIntire, K.R.: Lung Cancer Markers. In Sell, S. and Wahren, B. (Eds.): Human Cancer Markers. New Jersey, The Humana Press, Inc., 1982, pp. 359-380.

Princler, G.L., McIntire, K.R., and Braatz, J.A.: Identification and purification of a human lung tumor-associated antigen from primary lung tumor. Cancer Res. 42: 843-848, 1982.

Braatz, J.A., Scharfe, T.R., Princiler, G.L., and McIntire, K.R.: Characterization of a human lung tumor-associated antigen and development of a radioimmunoassay. Cancer Res. 42: 849-855, 1982.





# 1. BIOCHEMISTRY

- R01-CA-14185      Modified Nucleosides in Cancer and Normal Urines  
Girish Chheda                  Roswell Park Memorial Institute
- R01-CA-14380      Pancreatic Ductal Versus Duodenal Secretions  
Thomas T. White                  University of Washington
- R01-CA-19606      Serum Urine and CSF RNases in Health and Disease  
Charles A. Dekker                  University of California, Berkeley
- R01-CA-22599      Programs of Normal and Malignant Lymphocytes  
Allen E. Silverstone                  Sloan-Kettering Institute for Cancer Research
- R01-CA-22885      Lung Cancers and Carcinomatous Neuromyopathies  
Koichi Ishikawa                  University of Southern California
- R01-CA-23945      Assessment of Malignancy in Human Chondrosarcomas  
Lawrence C. Rosenberg                  Montefiore Hospital and Medical Center
- R01-CA-25016      Human Lung Neoplasms: Applications of Enzyme-Patholgy  
Olga Greengard                  Mount Sinai School of Medicine
- R01-CA-25210      Origins of Urinary Nucleosides in Tumor Tissue  
Ernest Borek                  AMC Cancer Research Center
- R01-CA-25376      Development of Serum Nuclease Isozyme Test for Cancer  
Kwan C. Tsou                  University of Pennsylvania
- R01-CA-25548      Isozymes of Human Tumor Cells In Vitro and In Vivo  
Jorgen Fogh                  Sloan-Kettering Institute for Cancer Research
- R01-CA-29056      Large Bowel Cancer & Colonic Microbial Metabolism  
David A. Mastromarino                  University of Texas
- R01-CA-29062      Vasoactive Intestinal Peptide, Leukocytes, Leukemia  
Mary S. O'Dorisio                  Ohio State University
- R01-CA-30627      In Vivo Release of Transformed Cell-Specific Proteins  
Thomas E. Webb                  Ohio State University
- R01-CA-30667      A Study of Cancer Associated Colonic Mucin  
Clement R. Boland                  University of California, San Francisco
- R01-CA-30687      Progesterone-Specific Protein in Endometrial Secretions  
George S. Richardson                  Vincent Memorial Hospital
- R01-CA-31187      Biochemical and Clinical Application of Acid Phosphatase 5  
Kwok-Wai Lam                  Albany Medical College
- R01-CA-31218      Thyrotropins from Tumors of Trophoblastic Origin  
Syed M. Amir                  Beth Israel Hospital

- R01-CA-32585      Creatine Kinase BB as a Tumor Marker  
                   Gerald B. Dermer                      University of North Carolina
- R01-CA-33615      Multiple Forms of HGH: Measurements and Actions  
                   Willard Vander Laan                      Whittier Institute for Diabetes & Endocrinology

## 2. IMMUNODIAGNOSIS

- P01-CA-04486      Pathology of the Digestive Tract Mucous Membrane  
                   Norman Zamcheck                      Boston City Hospital
- R01-CA-17742      Radiological Localization of Human Tumors  
                   David M. Goldenberg                      University of Kentucky Medical School
- R01-CA-19613      Ectopic Hormones in Small Cell Carcinoma of the Lung  
                   William G. North                      Dartmouth College
- R01-CA-21399      Binding of Bacteria to Normal & Luekemic Lymphocytes  
                   Marius Teodorescu                      University of Illinois Medical Center
- R01-CA-22141      Protein SAA in Neoplastic Disease  
                   Merrill D. Benson                      Indiana University School of Medicine
- R01-CA-22595      Detection of Medullary Thyroid Cancer in Families  
                   Charles E. Jackson                      Henry Ford Hospital
- R01-CA-24376      Immunological Heterogeneity of CEA  
                   James F. Primus                      University of Kentucky
- R01-CA-24645      Significance of GP48 in Diagnosis of Breast Cancer  
                   Zoltan A. Tokes                      University of Southern California
- R01-CA-25088      Human Ribonucleases and Cancer  
                   Dohn G. Glitz                      University of California, Los Angeles
- R01-CA-25338      Thyroglobulin Radioimmunoassay in Patients with Thyroid Cancer  
                   Merl A. Charles                      University of California, Irvine
- R01-CA-25381      Tyrosinase as a Marker for Human Malignant Melanoma  
                   Kenji Nishioka                      University of Texas System Cancer Center
- R01-CA-25584      Clinical CEA-Tumor Radioimmunodetection  
                   David M. Goldenberg                      University of Kentucky
- R01-CA-26246      Assay of Human Tumor or Organ-Associated Antigens  
                   Calvin A. Saravis                      Boston City Hospital
- R01-CA-26948      Nuclear Antigens as Markers in Hematopoiesis  
                   Robert C. Briggs                      Vanderbilt University
- R01-CA-27081      Immunodiagnosis of Mesothelioma  
                   Gurmukh Singh                      University of Pittsburgh

R01-CA-27213      Detection of Urogenital Normal and Neoplastic Antigens  
                  Robert W. Green                      Duke University Medical Center

R23-CA-27623      Characterization of Prostate Cell Plasma Membranes  
                  James J. Starling                      Eastern Virginia Medical Authority

R01-CA-28305      Possible Systemic Signals for Tumor  
                  Earl M. Ritzi                      University of Tennessee Center for Health Sciences

R01-CA-28462      Radiolabeling of Tumor Antibodies  
                  William C. Eckelman                      George Washington University

R01-CA-29225      Clinical Application of Esterase, a Monocyte Marker  
                  Kwok-Wai Lam                      Albany Medical College

R01-CA-29552      Differential Counting of Lymphocyte Subpopulations  
                  Marius Teodorescu                      University of Illinois Medical Center

R01-CA-29639      Tumor Imaging with Radiolabeled Monoclonal Antibody  
                  Steven M. Larson                      VA Medical Center

R01-CA-30019      Purification of Tumor Antigens of Defined Specificities  
                  Rishab K. Gupta                      UCLA Center for Health Sciences

R01-CA-30255      Immunolocalization of Human Malignant Tumors  
                  Elliott Alpert                      Baylor College of Medicine

R01-CA-31236      AFP/HCG Radioimmunodetection in Testicular Cancer  
                  David M. Goldenberg                      University of Kentucky

R01-CA-31701      Monoclonal Antibodies in Diagnosis & Prognosis of Cancer  
                  Darwin O. Chee                      City of Hope National Medical Center

R01-CA-31762      Immunologic Diagnosis of Myeloblastic Leukemia  
                  Robert N. Taub                      Columbia University School of Medicine

R01 CA-32245      Detection & Characterization of Mesenchymal Antigens  
                  Yashar Hirshaut                      Sloan-Kettering Institute for Cancer Research

### 3. CYTOLOGY

R01-CA-13271      Automated Cancer Cell Diagnosis by the TICAS Method  
                  George Wied                      University of Chicago

R01-CA-17353      Marrow Culture Studies in Human Myeloid Leukemias  
                  Malcolm A. Moore                      Sloan-Kettering Institute for Cancer Research

R01-CA-22942      Differentiation of Cultured Leukemic Cells  
                  Sandra R. Wolman                      New York University

R01-CA-23393      Flow Analysis of Human Malignant Lymphoid Cells  
                  Raul C. Braylan                      University of Florida

R01-CA-24466      Ultrafast Scanner Microscope in Laboratory Automation  
Roland V. Shack      University of Arizona

R01-CA-24932      Microspectrophotometric Nuclear DNA Study of Gynecologic Cancers  
Yao S. Fu      Case Western Reserve University

R01-CA-25348      Flow Cytometry/Autoradiography Monitoring of Leukemia  
Michael Andreeff      Sloan-Kettering Institute for Cancer Research

R01-CA-26863      Identification of CEA in Cytology Specimens  
Robert R. Pascal      St. Luke's-Roosevelt Institute for Health Sciences

R01-CA-27123      Application of Fluorescent Probes to Clinical Cancer  
Jay E. Valinsky      Rockefeller University

R01-CA-27283      Early Detection of Transformed Cells  
Susan P. Hawkes      Michigan Molecular Institute

R01-CA-27313      A Search for Pre-neoplastic Cell Markers in Sputum  
Stanley Greenberg      Baylor College of Medicine

R01-CA-28704      Chromatin Probes for Distinguishing Malignant Cells  
Zbigniew Darzynkiewicz      Sloan-Kettering Institute for Cancer Research

R01-CA-28706      Cell Positioning System: Development and Use in Cancer  
Harry W. Tyrer      Cancer Research Center

R01-CA-28724      Biophysical Probes for Malignant Cells  
Paul Todd      Pennsylvania State University

R01-CA-28770      Biophysical Probes for Automated Cytology  
Kwan C. Tsou      University of Pennsylvania

R01-CA-28771      Cytology Automation  
Barthel Barlogie      M. D. Anderson Hospital and Tumor Insitute

R01-CA-28833      Cytologic Characterization of Rat Urothelium  
Ian T. Young      Lawrence Livermore Laboratory

R01-CA-28886      Indexed Cell Sorting  
Phillip Dean      Lawrence Livermore Laboratory

R01-CA-28921      Merocyanine Dyes as Leukemia-Specific Probes  
Robert A. Schlegel      Pennsylvania State University

R13-CA-29859      Eighth Conference on Analytical Cytology  
Myron R. Melamed      Sloan-Kettering Institute for Cancer Research

R01-CA-30148      Development of Lanthanide Fluorescent Stains  
Lidia M. Vallarino      Virginia Commonwealth University

R01-CA-30582      Multistage Slit-Scan Prescreening System  
Leon L. Wheelless      University of Rochester Medical Center



R01-CA-31049      Clinical Test for Automated Prescreening Device  
George L. Wied      University of Chicago

R01-CA-31718      Automated Cytology Prototype Development  
Kenneth R. Castleman      California Institute of Technology

R01-CA-32314      Fluid Cell Sorter  
Leon L. Wheelless      University of Rochester

R01-CA-32345      Computer Image Analysis of Cells in Urothelial Cancer  
Leopold G. Koss      Montefiore Hospital and Medical Center

R13-CA-32959      Ninth Conference on Analytical Cytology  
L. S. Cram      Los Alamos National Laboratory

R01-CA-33148      Multidimensional Slit-Scan Detection of Bladder Cancer  
Leon L. Wheelless      University of Rochester

#### 4. PATHOLOGY

R01-CA-14264      Pathology of Cell Differentiation in Leukemia  
Dorothy F. Bainton      University of California, San Francisco

R01-CA-21905      Pituitary Adenomas: Structure-Function Relations  
Calvin Ezrin      Cedars-Sinai Medical Center

R01-CA-22101      Study of Head and Neck Cancer by Serial Section  
John A. Kirchner      Yale University

R01-CA-26422      Clinico-Biologic Correlation in Lymphoma and Leukemia  
Henry Rappaport      City of Hope National Medical Center

R01-CA-29211      Immunohistologic Study of Uterine Cancer  
Clive R. Taylor      University of Southern California

#### 5. MULTIPLE DISCIPLINES

R01-CA-25582      Fluorescence Endoscopy and Photoradiation Therapy  
Oscar J. Balchum      University of Southern California



# CONTRACT RESEARCH SUMMARY

Title: Collection of Serial Serum Samples from Cancer Patients

Principal Investigators:

Drs. Carl Feit & Yashar Hirshaut

Performing Organization:

Memorial Hospital for Cancer and  
Allied Diseases

City and State:

New York, NY

Contract Number: N01-CB-04350

Starting Date: 9/30/77

Expiration Date: 3/30/82

Goal: Collect serial serum specimens from four types of cancer patients and matched controls to provide a resource which will be used to expedite the clinical evaluation of new diagnostic tests for cancer.

Approach: Collect serial specimens from patients with melanoma, ovarian carcinoma, lung carcinoma, and Hodgkin's disease plus suitably matched patients with benign skin lesions, benign adnexal masses, a history of heavy smoking, and benign lymphadenopathy to serve as controls.

Progress: Patients with carcinoma of the lung, ovarian carcinoma, melanoma and Hodgkin's disease as well as matched control individuals with benign conditions have been followed from the time of diagnosis for 2 years or to the time of their death. Sera have been collected every 2-3 months. Additional samples were obtained before and after any significant change in the course of disease or treatment of those with malignancy. Appropriate documentation of clinical status at the time of each serum collection has been obtained. Sera are stored at -70°C. The clinical data are being entered into a computer for ready retrieval. At the time of termination of this contract on March 30, 1982, 326 subjects had been entered. Of these 165 were patients with malignancies and 161 control subjects. A total of 1,921 serum specimens are stored.

Project Officer: Dr. Bernice T. Radovich

Program: Diagnosis

FY 82 Funds: 0

## CONTRACT RESEARCH SUMMARY

Title: Biomedical Computing Software Support of Breast Cancer Treatment Program

Principal Investigator:	Ms. Marlene Dunsmore
Performing Organization:	Information Management Services, Inc.
City and State:	Bethesda, MD

Contract Number: N01-CB-14339

Starting Date: 3/31/81

Expiration Date: 3/30/83

Goal: To increase the usefulness of the data generated in projects sponsored by the Breast Cancer Task Force Treatment Program and in other designated projects related to the treatment of human breast cancer.

Approach: A central file was developed by the initial contractor for three areas of study: (1) surgical adjuvant therapy, (2) tumor biomarkers, and (3) estrogen receptor assays. The file allows comparisons of the results from various studies and provides a data base from which material can be quickly and conveniently retrieved. This data file is also intended for testing new ideas, identifying groups of subjects suitable for more detailed study and for preparing reports to the medical community and the general public. The file is not intended to duplicate those at individual institutions, but to prepare data for analyses that are not possible at the individual institutions.

Progress: Communication with the collaborating institutions has been maintained. The main file update system modifies clinical history files with new information and continues to edit for data consistency. Data concerned with treatment, histopathology, survival, and estrogen receptor status have been stored on almost 3000 patients. This data base is available for a variety of analyses concerned with receptor vs. clinical course correlations such as tumor recurrence rates and tumor response to endocrine and cytotoxic therapy. Underway is a feasibility study for comparing data across four adjuvant therapy trials. In support of the Biological Markers Project, background and clinical data have been gathered for 17,500 blood collections from three institutions. Benign tumor and metastatic cancer follow-up information continues to be submitted. Although the availability of the sera has not yet been officially announced, several requests have been received and approved. The Data Center has prepared the lists of coded samples and evaluated the laboratory results submitted by the investigators.

Project Officers: Mary E. Sears, M.D.; Ihor J. Masnyk, Ph.D.  
Program: Diagnosis  
FY 82 Funds: \$157,899

CONTRACT RESEARCH SUMMARY

Title: Human Tumor Cell Line Bank for Diagnostic Studies

Principal Investigator: Dr. Robert J. Hay  
Performing Organization: American Type Culture Collection  
City and State: Rockville, MD

Contract Number: N01-CB-14351

Starting Date: 9/29/81

Expiration Date: 9/28/86

Goal: The objectives of the program are to acquire, characterize, catalog, store and distribute a variety of cell lines having special utility for research in tumor diagnosis. Well characterized lines from solid tumors as well as from normal tissues will be included. Information concerning properties and utility of these lines will also be provided to recipient investigators.

Approach: Cell lines in the existing bank will be expanded from token holdings to produce seed and distribution stocks. These will be partially recharacterized using published ATCC procedures. Initially, standard tests for absence of microbial contamination will be applied, and species verification will be accomplished by assay for the isoenzymes of glucose-6-phosphate dehydrogenase, lactic dehydrogenase and nucleoside phosphorylase. Distribution of the new stocks will begin concomitantly with further testing (for karyology, surface antigens, isoenzyme profiles, etc.) and accessioning of new lines.

Progress: Ad-Hoc advisors to the Tumor Cell Line Bank reviewed the cell line holdings in the former bank and a group of 118 were selected for transfer from the total of 443 lines listed previously. Characteristics of the selected lines arranged by tumor site and by ATCC number were prepared for distribution. Efforts to compose a new catalog were initiated.

Transfer of the inventory was completed early in January, 1982, and the tasks of expanding and partially recharacterizing the resultant stocks were started. To date, 27 of the lines have been expanded and partially recharacterized and 38 are being expanded. Distribution has been resumed and should be normal by June, 1982.

Project Officer: Dr. Bernice T. Radovich  
Program: Diagnosis  
FY 82 Funds: \$188,221



# CONTRACT RESEARCH SUMMARY

Title: Data Management System for NCI Serum Panels

Principal Investigators:

Dr. Lee A Richman and  
Dr. C.M. Dayton

Performing Organization:

Ebon Research Systems

City and State:

Rockville, MD

Contract Number: N01-CB-14359

Starting Date: 8/08/77

Expiration Date: 3/07/83

Goal: To provide data management and statistical programming support for research projects being conducted by the Diagnosis Program in order to determine which serum tests are best able to detect early stages of cancer.

Approach: To perform statistical analyses of laboratory and clinical data from approximately two NCI serum panels per month, and to prepare summary reports of the results.

Progress: During the past year improvements were made in the analytic approach for evaluating cancer panels, particularly those panels containing multiple assays. Routine screening is done for age and sex imbalance between clinical groups; the Mantel-Haenszel procedure is applied when a number of previous cancer patients exhibit no evidence of disease, and receiver operator curves are supplied (plotting "1.0 - Sensitivity" versus "1.0 - Specificity"). For multiple assays, an "either-or" rule was adopted (i.e., if either assay is positive for a given case, the case is considered positive), and logistic regression and discriminant analyses are carried out. The ICDO coding system has been adopted for coding cancer cases, and a modification of this system has been applied for coding benigns.

Project Officer: Dr. Bernice T. Radovich

Program: Diagnosis

FY 82 Funds: \$79,032

CONTRACT RESEARCH SUMMARY

Title: Carcinoembryonic Antigen in the Diagnosis of Bowel Cancer

Principal Investigator:	Dr. Vay Liang W. Go
Performing Organization:	Mayo Foundation
City and State:	Rochester, MN

Contract Number: N01-CB-23854

Starting Date: 6/15/72

Expiration Date: 6/14/82

Goal: To determine the usefulness of CEA in the diagnosis of bowel cancer.

Approach: Measure CEA levels in blood stored at NCI-Mayo Clinic Human Serum Bank (CB-84258), for the Diagnosis Program of the Division of Cancer Biology and Diagnosis.

Progress: Serum levels have been determined in 4,586 patients from the NCI Serum Bank (CB-84258). A commercial Enzyme Immunoassay Kit is used. The samples have been run at 25% of commercial cost. The CEA stability of selected serum samples provides a quality control on the adequacy of the storage conditions and absence of bacterial or enzymatic degradation in the NCI Serum Bank. This contract will be phased out on June 15, 1982.

Project Officer: Dr. K. Robert McIntire  
Program: Diagnosis  
FY 82 Funds: 0

# CONTRACT RESEARCH SUMMARY

Title: Slit-Scan Technique as Automated Cancer Prescreening System in Cytology

Principal Investigator:  
Performing Organization:

Dr. Leon L. Wheeless, Jr.  
University of Rochester  
Medical Center  
Rochester, NY

City and State:

Contract Number: N01-CB-33862

Starting Date: 3/19/73

Expiration Date: 5/19/82

Goal: Automation of clinical cytologic screening and diagnosis.

Approach: A benchmark study of the X-Y-Z system will be performed to: (1) determine system characteristics including rate of remaining false alarms; (2) determine the alarm rate for abnormal specimens, and (3) determine specimen false positive and false negative rates in a preliminary clinical trial.

Progress: The X-Y-Z Slit-Scan Flow System was fabricated and testing initiated. A spectrum of normal and abnormal clinical specimens were used to evaluate system performance characteristics and establish a flow data base. Preliminary results were very encouraging. Four hundred thirty-eight clinical specimens were analyzed from January 1981 through January 1982 in a single blind study. The false negative rate on 92 abnormal specimens representing the full spectrum of abnormality was 2.4 percent. Abnormal specimen types included 22 dysplasias, 6 carcinoma-in-situ, 41 squamous cell carcinomas, 17 adenocarcinomas, 3 adenosquamous carcinomas, 2 leiomyosarcomas, and 1 mixed mesodermal sarcoma. From this spectrum of abnormal specimen types only two slight dysplasias were misclassified. The remaining 37 specimens in the study contained atypical squamous cells. The instrument in its present configuration appears sensitive to the entire spectrum of abnormality existing in the female genital tract and will classify as abnormal any specimen containing at least 0.1 percent abnormal cells.

An alternate system concept has been tested to provide similar multidimensional slit-scan information from a less complex instrument. This new instrument has been shown feasible and will additionally provide a significant increase in system throughput. Fabrication and clinical testing of this new instrument, together with parallel testing with the X-Y-Z system, has been funded under grant #R01-CA-30582.

Project Officer: Dr. Bill Bunnag  
Program: Diagnosis  
FY 82 Funds: 0

CONTRACT RESEARCH SUMMARY

Title: Statistical Center for Cooperative Lung Cancer Groups

Principal Investigator:

Dr. C. Ralph Buncher

Performing Organization:

University of Cincinnati

Medical Center

City and State:

Cincinnati, OH

Contract Number: N01-CB-43868

Starting Date: 6/10/74

Expiration Date: 2/28/83

Goal: To collect and analyze information from the three clinical centers in the lung cancer screening program (directed toward the detection of early lung cancer in high-risk patients) to ascertain whether this screening program has reduced mortality and morbidity.

Approach: Procedures have been established with each of the centers and agreement has been reached concerning the common data base for this study. Data are routinely monitored by the Central Statistical Group (CSG), translated into a single computer data base in Cincinnati, and analyzed to provide the combined collaborative information as well as comparative information. Reports are made quarterly. The CSG will continue to search the data from this study for important and statistically significant findings.

Progress: A common data base for this three clinical center study has been established. Translation and reporting systems for each of the clinical centers have been created and analyses of collaborative results to date have been provided to the NCI and participating clinical centers. Intake of subjects has been completed. Adherence and compliance with the screening program has been a focus. Now the active screening is winding down to completion. Thousands of subjects have completed the screening program. Survival analysis efforts are being made to obtain meaningful results with respect to mortality differences in the screening groups at as early a time as feasible. Regular Mortality Review Committee conferences are held to discuss the death certificate and best information cause of death for each man in the study who has died.

Project Officer: Dr. Bill Bunnag

Program: Diagnosis

FY 82 Funds: \$158,542

## CONTACT RESEARCH SUMMARY

Title: Lung Cancer - Early Detection, Localization and Therapy

Principle Investigator: Dr. Myron R. Melamed  
Performing Organization: Memorial Hospital for Cancer  
and Allied Diseases  
City and State: New York, NY

Contract Number: N01-CN-45007

Starting Date: 9/01/73

Expiration Date: 7/31/88

Goal: To evaluate sputum cytopathology as a supplement to annual chest x-rays in detecting pulmonary neoplasms at an "early stage" and to evaluate the efficacy of techniques for prompt localization of radiologically occult lung cancer (e.g., before progression to x-ray positive); in general, to evaluate the efficacy of such screening to reduce lung cancer mortality.

Approach: Over a 3-year period, 5,000 test subjects and 5,000 control subjects have been entered into this study. Each subject will receive active screening for at least 5 years, all followed for an additional 5-year period.

Progress: Recruitment for the Memorial Sloan-Kettering Cancer Center National Lung Program was completed in January 1978. Total enrollment is 10,040. 4,968 participants were randomly assigned to study group (A), receiving annual chest x-rays and 4-monthly sputum; and 5,072 to control group (B), receiving annual x-rays only. There have been a total of 257 confirmed lung cancers identified so far, 127 in group A and 130 in group B. The principal mode of detection in group A was cytology in 23 cases, x-ray in 55 cases and both techniques in 13 cases. Of the 23 cases detected by cytology, 14 were Stage 0 or I; of the 68 cases detected by radiology (or radiology and cytology) in group A and the 82 cases detected by radiology in group B, there were 79 in Stage I. There were 84 interval cancers diagnosed following symptoms or signs or by x-rays taken outside of the routine screening program; only 17 of these were Stage I, and 23 were oat cell cancer.

Among the 127 lung cancers appearing in group A, 30 were prevalence cancers for a prevalence rate of 6.0/1,000 and 97 were incidence cancers for an incidence rate of 3.5/1,000/year. In group B, the 130 lung cancers included 23 prevalence for a prevalence rate of 4.6/1,000 and 107 incidence cases, for an incidence rate of 3.8/1,000/year.

The prevalence studies are now nearing completion. The number of lung cancers that were completely resected among the prevalence cases was 29 of 53 (55%); for all the cases it is 135 of 257 (53%). The survival rates are closely related to stage of disease at time of detection and treatment. There were 110 of the total of 257 lung cancers detected in Stage 0 & I, and they had an overall survival of 70% at 5 years. The Stage II and III lung cancers have approximately 5% survival at 5 years. Adenocarcinoma and epidermoid carcinoma have the best survival; oat cell carcinoma has the poorest survival.

Project Officer: Dr. Bill Bunnag  
Program: Diagnosis  
FY 82 Funds: 675,000



## CONTRACT RESEARCH SUMMARY

Title: Lung Cancer Control: Detection and Treatment

Principal Investigator:	Dr. John K. Frost
Performing Organization:	Johns Hopkins University
City and State:	Baltimore, MD

Contract Number: N01-CN-45037

Starting Date: 1/01/73

Expiration Date: 7/15/88

Goal: To determine the effect on mortality from lung cancer of early detection of this disease by sputum cytology followed by surgical removal of the tumor.

Approach: The study consists of males who were 45 years or older and had smoked a pack or more of cigarettes a day. Individuals responding to invitation were randomly assigned to one of two groups: the "X" group receives an annual chest x-ray only; the "CX" group receives an annual chest x-ray, an annual sputum induction plus a sputum cytologic examination every four months. All subjects will be followed after final screening to determine actuarial survival.

Progress: 5,161 men were randomized into the "X" group and 5,226 into the "CX" group. To date, 1130 have died, while 2240 have withdrawn from the study or moved from the area. Currently 7017 remain as active participants, 3573 in the "X" group and 3444 in the "CX" group. At the initial screening, 78 cancers were detected (40 in the X group and 38 in the CX group) for a "detected prevalence" of 7.5 per 1000. Following an initial screening, a total of 290 cancers appeared (152 in the X group and 138 in the CX group) for an "annual incidence" of 5.1 per 1000. In the CX group, 52 (65%) incidence cases were detected at AJC Stage I, 10 (19%) of these were in situ and 3 (6%) were microinvasive, and 39 (75%) invasive; 58 additional cases occurred between screenings, 10% at Stage I. In the X group, 42 (53%) incidence cases were detected at AJC Stage I, all invasive; 73 additional cases occurred between screenings, 19% at Stage I. Among the 138 cases occurring in the CX group after initial screening, 59 (43%) were resected; 61 (40%) of the 152 incidence cases occurring in the X group were resected. In the screened population, 368 total lung cancers have appeared: 192 in the X group and 176 in the CX group. Among the 192 cases of lung cancer found in the X group, 130 (68%) have died; of the 176 lung cancer cases in the CX group, 105 (60%) have died. Although the mortality rates of cancers detected at first screening and the mortality rates of cancers detected at subsequent screening show reduced mortality for the CX group, separately these comparisons fail to reach statistical significance. However, when the total number of lung cancer deaths which have occurred are combined over all age groups and appropriately weighted the overall mortality due to lung cancer among the CX group is significantly lower than that of the X group. With no statistical CX-X difference in the incidence of lung cancer, the observation of a reduced lung cancer mortality in the CX group seems likely to be due to the effect of cytologic screening, early detection and therapy. However, it must be realized that the entire benefit of cytologic screening seems to be confined to only one cell type (squamous).

Project Officer: Dr. Bill Bunnag  
Program: Diagnosis  
FY 82 Funds: \$626,045

# CONTRACT RESEARCH SUMMARY

Title: Screening Technique for Blood in Stool to Detect Early Cancer of Bowel

Principal Investigator:

Dr. Victor A. Gilbertsen

Performing Organization:

University of Minnesota Health  
Sciences Center

City and State:

Minneapolis, MN

Contract Number: N01-CB-53862

Starting Date: 6/30/75

Expiration Date: 6/29/83

Goal: To demonstrate a significant reduction in mortality from colorectal cancer between the screened and the control groups by employing the Hemoccult (R) form of the guaiac test for occult stool blood in combination with a diagnostic protocol to localize the source of bleeding.

Approach: Forty-five thousand participants between the ages of 50 and 80 years residing in the state of Minnesota were randomly allocated to three groups on the basis of age, sex and geographic location in the state. Slides are completed after the observance of a meat-free diet with suggested high fibre content. One group completes the slides yearly, another every other year, and a third is unscreened, serving as controls. Each slide set consists of 6 slides to accommodate two smears from each of three consecutive stools. Participants submitting samples positive for blood receive a diagnostic examination at the University of Minnesota Medical Center. The examination includes a complete history and physical examination, upper G.I. x-ray study (and gastroscopy if indicated), proctoscopy and colonoscopy (and barium enema if indicated).

Progress: The screening protocol designed to provide the initial prevalence screen and four annual and two biennial screens will be completed in early 1983. Follow-up procedures have been implemented to procure post-screening data from all living participants in the second phase of the project. All deaths are investigated to determine the presence of malignancy in the history.

A Deaths Review Committee consisting of oncologists and surgeons from outside of the University of Minnesota has routinely reviewed documentation and pathology reports (the latter by the project pathologist) and classified all deaths by the relationship of cancer of the colon and rectum to that death, i.e., determined whether the death is from or with colorectal cancer. This evaluation will continue through the follow-up phase of the project and provide the comparative mortality from colorectal cancer in the control and screened groups.

Project Officer: Dr. K. Robert McIntire

Program: Diagnosis

FY 82 Funds: 800,000

# CONTRACT RESEARCH SUMMARY

Title: Detection and Localization of Early Lung Cancer

Principal Investigator:  
Performing Organization:  
City and State:

Dr. Robert S. Fontana  
Mayo Foundation  
Rochester, MN

Contract Number: N01-CB-53886

Starting Date: 9/27/74

Expiration Date: 9/26/88

Goal: To test new methods of diagnosis of early lung cancer and to assess survival of patients with lung cancer detected by these methods.

Approach: A study population of 9,211 subjects was obtained following initial screening of 10,935 non-volunteers. Candidates were Mayo Clinic outpatients who were men, 45 years old or more, smoking one pack of cigarettes or more daily. None of those accepted into the study had a history or suspicion of respiratory cancer on entry into the Clinic. Initial screening consisted of chest radiographs, 3-day sputum cytology tests and lung health questionnaires. This screening yielded 91 unsuspected ("prevalence") lung cancers, 17 detected by cytology alone, 59 by radiography alone, and 15 by both tests. Radiographically "occult" cancers were localized bronchoscopically. The 9,211 study subjects who had "negative" initial screens were randomized into a close-surveillance group of 4,618, for whom rescreening every 4 months was urged, and a standard surveillance ("control") group of 4,593 advised, but not reminded, to undergo rescreening once a year. A population of postsurgical AJC stage I lung cancer patients is also being evaluated, as is hematorporphyrin derivative (HpD) as a technique for "mapping" and treating carcinoma in situ.

Progress: As of January 1, 1982, the 4-monthly surveillance group and the control group had each been observed 30,000 man-years, and 341 new ("incidence") cases of respiratory cancer had been detected. Of these, 69 involved the upper airway and 272 the lungs. In the 4-monthly surveillance group there were more cancers, more early-stage cancers, more resectable cancers and better survivorship than in the control group. However the number of advanced cancers and lung cancer deaths was approximately equal in the two groups. There is a delay in obtaining information concerning the control group, which is contacted only once a year. Thus simultaneous comparisons of mortality favor the controls. This disparity should be resolved within three years. As incidence cases continue to accrue, more detailed analyses of data are feasible. Attention is currently being directed toward evaluating the results of screening by cell type of tumor and by modality of detection. The HpD study has detected several squamous cancers that were both radiographically and endoscopically occult.

Project Officer: Dr. Bill Bunnag  
Program: Diagnosis  
FY 82 Funds: 500,000

## CONTRACT RESEARCH SUMMARY

Title: Screening for Medullary Carcinoma of the Thyroid

Principal Investigator:	Dr. Samuel A. Wells, Jr.
Performing Organization:	Duke University Medical Center
City and State:	Durham, NC

Contract Number: N01-CB-63994

Starting Date: 6/15/76

Expiration Date: 11/30/81

Goal: To establish a periodic screening program for relatives of patients with medullary thyroid carcinoma (MTC).

Approach: A cohort of patients found in 13 kindreds, in whom MTC was proven histologically was large enough to offer a statistically significant number of family members. Those family members were screened for serum calcitonin levels by radioimmunoassay methods in order to detect MTC in its earliest stage of development. A long-term follow-up program will be conducted in these families to determine the natural history of the disease and the effectiveness of surgical and possible chemical therapy.

Progress: A study of approximately 800 subjects from 13 kindreds with multiple endocrine neoplasia, type II (medullary thyroid carcinoma [MTC]), pheochromocytoma and hyperparathyroidism) has completed its sixth year. They have demonstrated that the combined infusion of calcium gluconate (2 mg/kg/1 min) followed by pentagastrin (0.5 µg/kg/5 sec) is the most sensitive method of stimulating calcitonin (CT) release from MTC cells. This combined infusion serves as the most efficient method for establishing the early diagnosis of MTC. Over the last 2 years they have identified two kindreds who inherit only MTC with none of the extrathyroidal manifestations of multiple endocrine neoplasia (MEN), types IIa and IIb. Furthermore, the MTC in these patients appears to be less aggressive than the MTC occurring in subjects with MEN IIa or subjects with MEN IIb.

We continue to evaluate chemotherapeutic agents in the treatment of patients with metastatic MTC. Thus far, of several chemotherapeutic agents, none has proven of benefit with the possible exception of adriamycin in two patients.

Project Officer: Dr. K. Robert McIntire  
Program: Diagnosis  
FY 82 Funds: 0

## CONTRACT RESEARCH SUMMARY

Title: Digital Image Processing Techniques in Cytology Automation

Principal Investigator:  
Performing Organization:

Dr. Kenneth R. Castleman  
JPL-California Institute of  
Technology  
Pasadena, CA

City and State:

Contract Number: Y01-CB-70314

Starting Date: 9/19/77

Expiration Date: 11/30/81

Goal: Identification of the best performing image analysis algorithms and estimations of the expected performance and cost per specimen of an automated cervical cancer pre-screening system using these algorithms.

Approach: The research will be done to determine whether or not the current state of the art in pattern recognition is adequate to support the development of an economically viable cytological screening instrument based on single cell classification. Specimen preparation protocol will be optimized so as to produce acceptable single cells for digitization. A large number of digitized images will be accumulated in a library for a subsequent feature extraction program to produce a feature data base. A series of classification experiments will be run on the feature data base to select the subset of cell measurements that yield the best overall performance. An analysis will produce estimates of the cost and performance using these techniques in practice.

Progress: A new specimen preparation protocol has been developed. Over 10,000 digitized cell images were acquired and classification algorithms were developed and tested. New statistical methods for optimal specimen classification and analysis have been developed. The analysis of a cascade of a cell classifier followed by a specimen classifier has provided quantitative criteria for evaluating all classifier effectiveness as part of an overall system. This analysis has also provided guidelines for tuning the parameters of the cell classifier. A cost/utility analysis has indicated that the cell classification algorithms chosen can operate with error rates that are adequate for routine screening.

The second phase of the study has been completed. This involves the development of fully automatic cell location, measurement, and classification algorithms that operate wholly without human intervention. Cost/utility analysis of the resulting data base indicates that cell classification performance is still adequate to support cost-effective automated screening for cervical cancer.

Project Officer: Dr. Bill Bunnag  
Program: Diagnosis  
FY 82 Funds: 0



CONTRACT RESEARCH SUMMARY

Title: Diagnostic Applications of Antibodies to Melanoma

Principal Investigator:	Dr. Peter Hersey
Performing Organization:	Kanematsu Institute
City and State:	Sydney, Australia

Contract Number: N01-CB-74120

Starting Date: 9/30/77

Expiration Date: 9/29/82

Goal: Determine whether there is a correlation between the clinical course of melanoma growth and levels of serum antibodies.

Approach: Employ lymphocyte-dependent cytotoxic antibody (LDA) assays.

Progress: From September 1, 1981 through January 1, 1982, 15 new patients with primary melanoma and 10 patients with other cancers were entered into the study. Total patients now entered is 349 (this excludes 94 patients studied at the time of their first recurrence). No further patient accrual is planned as the follow-up period to the end of the study in September, 1982 would be too short for meaningful analysis. Of those studied, 3 had transient leukocyte dependent antibody (LDA) detected against cultured melanoma cells and 3 had LDA detected in the "unblocked" IgG fraction of sera. The remainder were negative against all cell lines. Since September 30, 1981, LDA assays have been continued on serum samples collected during follow-up of patients and clinical progress recorded. No significant differences in recurrence rate in relation to LDA status is yet apparent. Both clinical and laboratory data are entered into the computer for final analysis. Analysis of the specificity of the sera after absorption on fetal brain tissue is still in progress. Complete details on all patients in the study will be included in the final report.

Project Officer: Dr. Bernice T. Radovich  
Program: Diagnosis  
FY 82 Funds: 0

## CONTRACT RESEARCH SUMMARY

Title: Biologic Markers in Breast Cancer: Patient Resource

Principal Investigator: Dr. Ned D. Rodes  
Performing Organization: Cancer Research Center  
City and State: Columbia, MO

Contract Number: N01-CB-74138

Starting Date: 9/1/77

Expiration Date: 8/31/83

Goal: To serve as a Breast Cancer Task Force specimen resource for blood from breast cancer patients and controls to be used in a search for and verification of new breast cancer markers.

Approach: 30 ml of blood are collected from volunteer Breast Cancer Detection Project and Women's Cancer Control Program patients after they have signed appropriate consent forms. In the event of subsequent malignant breast biopsy, a 30 ml specimen is also collected postmastectomy within three months. In addition, pre-biopsy and postmastectomy blood samples will be obtained from hospitalized ladies in the Columbia area who agree to participate in the program. Emphasis is now placed upon the collection of samples from symptomatic patients rather than specimen collection from normal volunteer screenees. Anniversary serum from malignant patients will be obtained for three, possibly five years, and annual clinical history from patients with benign biopsies. All blood will be stored at -70°C, and then shipped to NCI-designated serum storage facility with appropriate clinical data.

Progress: Since September 1980, the primary emphasis for the Cancer Research Center Serum Collection Unit has been to obtain serum samples preoperatively from patients with benign breast disease, and pre and postoperative malignant breast disease samples. Along with this, we have continued to collect serum samples from normal participants and annual followups. In the first 16 months after September 1980, the Cancer Research Center Serum Collection Unit collected 10,797 vials of serum from 933 participants. There were 18 "void" (broken tubes or hemolyzed specimens) for a net result of fully processed serum samples from 915 volunteers. This reflects a successful process rate of 98.1%. Our average vials of serum samples ratio was 11.8. A breakdown in each category is as follows: 63 preoperative carcinoma; 50 postoperative carcinoma; 57 preoperative benign; 368 controls; 210 malignant followups; 190 symptomatic category reflects participants from the Women's Cancer Control Program who had undiagnosed breast abnormalities at the time of serum collection. By maintaining careful followup of the volunteers, some of the symptomatic serums will later be placed in one of the above categories. When appropriate (malignant breast disease) postmastectomy serum samples will be obtained.

Project Officers: Ihor J. Masnyk, Ph.D.; Mary E. Sears, M.D.  
Program: Diagnosis  
Fy 82 Funds: \$57,000

CONTRACT RESEARCH SUMMARY

Title: Application of Digital Image Processing Techniques to Cytology Automation

Principal Investigator:

Dr. James W. Bacus

Performing Organization:

Rush-Presbyterian-St. Luke's  
Medical Center

City and State:

Chicago, IL

Contract Number: N01-CB-74190

Starting Date: 9/30/77

Expiration Date: 6/30/82

Goal: Development of optimal algorithms for cell classification applicable to an automated digital image processing system.

Approach: A comprehensive study will be conducted to determine single cell vs. specimen classification accuracies for image processing algorithms and for cytotechnologists. This will determine how well the machine algorithms are performing. Five experimental tasks will be done: 1) sample acquisition, 2) cell acquisition, 3) observer recognition, 4) cell classification algorithms developments, and 5) analysis and evaluation of results.

Progress: 1) Patient selection and sample acquisition have been completed. 2) Fabrication of the cell acquisition system is finished and all cells have been acquired. True classification of all cells was obtained by consensus of three cytotechnologists with access to full case information. 3) A preliminary experiment, in which 23 observers classified 1,650 cells, both with and without background information, resulted in the selection of a sub-set of 10 observers for the main experiment involving 6,375 cells, both with and without background. To date, three observers have completed this experiment, the remaining 7 observers have each completed a minimum of 3,600 cells. 4) Computer cell classification algorithms, based largely on texture analysis, have been developed. It has been found that they give results comparable to the best human observers, when provided with good manual segmentations. For a known cell recognition accuracy, and for a desired slide recognition accuracy, it is possible to calculate the number of cells which must be observed. 5) Data have been evaluated by Receiver Operating Characteristic (ROC) analysis, for the preliminary experiment, and also for the main experiment up to 2,700 cells. It has proved possible to obtain a complete ROC curve for each observer, by assigning levels to the different cell classifications. It appears that there is little increase in the classification accuracy of human observers when local background information is available.

Project Officer: Dr. Bill Bunnag

Program: Diagnosis

FY 82 Funds: 0

# CONTRACT RESEARCH SUMMARY

Title: Biological Markers in Breast Cancer: Patient Resource

Principal Investigator: Dr. Theodore Maycroft  
Performing Organization: Butterworth Hospital  
City and State: Grand Rapids, MI

Contract Number: N01-CB-74213

Starting Date: 9/15/77

Expiration Date: 9/14/83

Goal: To develop a Breast Cancer Task Force specimen resource for blood from breast cancer patients, benign disease patients and controls to be used in a search for and verification of new breast cancer markers.

Approach: Thirty milliliters of blood are collected prior to surgery from patients who are scheduled to undergo biopsy and/or primary surgery for breast lesions. Specimens are collected also in the early post-mastectomy period. Annual drawings are made on patients with malignant diagnoses. Patients with benign tumors are requested to complete annual questionnaires for a period of two years after biopsy. Serum specimens are stored at  $-70^{\circ}\text{C}$ , then shipped to an NCI designated blood bank facility with appropriate clinical data.

Progress: After a formal presentation to the surgery sections in each participating hospital, surgeons who perform 95% of all breast biopsies in these hospitals signed letters of agreement allowing their patients to enter directly into the study. Since the inception of the program, more than 2800 patients have been entered into the study. Approximately 30,000 vials containing serum specimens have been shipped to the central storage facilities at Mayo Clinic. Collections have been made on the annual anniversary of malignant patients and follow-up questionnaires have been completed on patients with benign lesions. This information has been forwarded to the Data Center. Fifteen months into the program, all participating patients and interested parties were invited to an informational update presentation provided by NCI project officers. The audience numbering close to 500 were congratulated for their participation and encouraged to continue their cooperative efforts. The participating surgeons and personnel from the collaborating hospitals have continued to give excellent cooperation with the biomarkers project staff.

Project Officer: Ihor J. Masnyk, Ph.D.; Mary E. Sears, M.D.  
Program: Diagnosis  
Fy 82 Funds: \$47,500

# CONTRACT RESEARCH SUMMARY

Title: Evaluation of Screening Methods for Endometrial Cancers

Principal Investigator: Dr. Leopold G. Koss  
Performing Organization: Montefiore Hospital  
and Medical Center  
City and State: Bronx, NY

Contract Number: N01-CB-84233

Starting Date: 9/30/78

Expiration Date: 8/31/82

Goal: To document the feasibility of screening asymptomatic women, age 45 and above, for endometrial carcinoma by cytologic techniques and direct endometrial sampling.

Approach: Two thousand nine hundred eighty-two women, age 45 and above, have been enrolled in the study. Each has filled out an extensive epidemiologic questionnaire and was asked to sign an informed consent. Cytologic samples were obtained from the vagina and the uterine cervix. The endometrium was sampled with two commercially available devices: Isaacs' Curity and Mi-Mark, assigned randomly by computer. A second annual examination has been administered to 1400 women, and it is planned to conclude this project by August 31, 1982.

Progress: Of the 2982 women enrolled, the endometrial sampling could not be secured in 247 for various reasons, such as refusal to sign informed consent, prior hysterectomy, or other medical reasons. Sampling could be attempted in 2587. In this group successful endometrial sampling was accomplished in 89.3% of the sample. Adequate laboratory samples were obtained in 85% of the sample. Moderate or severe pain during or after sampling was reported by about 20% of the examinees, with more discomfort reported with the MiMark sampler. The first examination identified 14 occult endometrial carcinomas in women ages 56 to 72. Three additional endometrial cancers were missed: two women became symptomatic and 1 was diagnosed on rescreening of a suspicious smear. Thus, the prevalence of endometrial carcinoma was 6.5/1000.

The results of the second annual rescreening of 1500 women were as follows: one endometrial carcinoma, and 3 endometrial hyperplasias. One carcinoma in situ and 1 hyperplasia were missed. Thus, the incidence of carcinoma of the endometrium was 1.3/1000.

Incidental findings included: one tubal carcinoma, 2 ovarian carcinomas, 3 carcinomas of the breast, 10 cervical intraepithelial neoplasias.

Project Officer: Dr. Bill Bunnag  
Program: Diagnosis  
FY 82 Funds: 0



# CONTRACT RESEARCH SUMMARY

Title: Maintenance of the NCI Serum Diagnostic Bank

Principal Investigator:	Dr. Vay Liang W. Go
Performing Organization:	Mayo Foundation
City and State:	Rochester, MN

Contract Number: N01-CB-84258

Starting Date: 9/30/78

Expiration Date: 11/30/82

Goal: To establish and maintain a bank of sera from patients with cancer, with benign diseases and from normal individuals, for evaluating immunodiagnostic tests of potential clinical usefulness in the diagnosis of cancer.

Approach: Collect and make necessary serum samples available for evaluation of immunodiagnostic tests for cancer. Serve as a central facility for storage of serum and plasma specimens collected by other contractors in the Diagnosis Program.

Progress: Through December 31, 1981 the Mayo central facility contained 270,557 vials of sera stored in -75°C freezers. This inventory includes blood collected from the University of Minnesota and the Philadelphia Geriatric Center for long-term storage. In addition the Mayo central facility has collected an additional 35,200 vials of sera in 1981 and has shipped out 2,699 vials to 25 investigators. Additional information regarding carcinoembryonic antigen (CEA) values (as requested) as well as clinical information on the sera have been provided to the investigators. Analyses of assay and clinical data for statistical significance in discriminating cancers from benigns and controls are performed by NCI contractor.

The availability of serum aliquots on each patient has enabled a multi-institution collaborative study to be designed. The study will determine to what extent the simultaneous assay of several unrelated tumor markers will improve the sensitivity and specificity of any of the markers used alone.

Project Officer: Dr. Bernice T. Radovich  
Program: Diagnosis  
FY 82 Funds: \$185,000

## CONTRACT RESEARCH SUMMARY

Title: Immune Assays for Enzymes and Isozymes in Cancer

Principal Investigator:	Dr. Joel Shaper
Performing Organization:	Johns Hopkins University
City and State:	Baltimore, MD

Contract Number: N01-CB-84260

Starting Date: 9/22/78

Expiration Date: 3/14/82

Goal: To develop immunodiagnostic assays for cancer using an enzyme or isozyme as an antigen and to look for a correlation of enzyme/isozyme serum levels with tumor burden.

Approach: Evaluate the immunodiagnostic significance of serum levels of UDP-galactosyltransferase in human breast and ovarian carcinoma.

Progress: Antisera directed against affinity-purified bovine UDP-galactosyl-N-acetylglucosamine transferase (GT) have been prepared in rabbits and immunologically characterized. These antisera have relatively high titered precipitating antibody to bovine GT as assessed by the Ouchterlony double diffusion technique and inhibition of enzymatic activity. The antisera are highly crossreactive with human GT found in both normal serum and ascitic fluid obtained from a patient with ovarian cancer as determined by direct precipitation and inhibition of enzymatic activity. This cross-reactivity has been exploited for the development of a sensitive and specific RIA for human serum GT with a detection level of 0.1-1.0 nanograms in the assay mixture.

A retrospective clinical study on patients with Stage IV breast carcinoma has been completed. These serum samples were initially collected and GT levels quantitated by both kinetic assay and RIA. In this limited study, GT levels by both procedures were in good agreement. Additionally, a prospective clinical study on individuals with breast carcinoma has been completed using a coded panel of serum samples. In contrast to the retrospective study, serum GT quantitation by RIA revealed no significant difference between the four diagnostic groups analyzed: (a) breast cancer, (b) benign breast disease, (c) normal age-matched females and (d) normal males. The reason for the discrepancy between these two studies is not immediately obvious. One problem may be that serum GT does not survive long-term storage. In this context it has been observed that repeated freezing and thawing of individual samples result in a significant decrease in GT levels as quantitated by kinetic assay.

Project Officer: Dr. Bernice T. Radovich  
Program: Diagnosis  
FY 82 Funds: 0

CONTRACT RESEARCH SUMMARY

Title: NCI Sera Bank Facility for the Breast Cancer Task Force

Principal Investigator:	Dr. Vay Liang W. Go
Performing Organization:	Mayo Clinic Foundation
City and State:	Rochester, MN

Contract Number: N01-CB-84296

Starting Date: 9/1/77

Expiration Date: 8/31/83

Goal: To establish and maintain a storage facility for serum specimens to be used by the Breast Cancer Task Force in a program designed to search for biological markers in breast cancer.

Approach: Serum specimens will be secured from breast cancer patients, benign disease patients, controls, and a screening population under separate contracts setting up a patient resource. The material will be processed, recorded and stored in -70° C deep freezers under easily retrievable conditions with all clinical data available. The sera will be used in the search for and verification of new breast cancer markers.

Progress: Collection and inventory methods have been developed. A special vial has been obtained and supplied to the collection areas. An operational shipping schedule has been established on a regular basis. Samples have been catalogued and systematically stored. Twenty-three freezers are occupied with the more than 175,000 serum samples. Thirteen coded serum panels have been sent out to investigators in the United States and abroad. All arrived in good condition.

Project Officer: Ihor J. Masnyk, Ph.D.; Mary E. Sears, M.D.  
Program: Diagnosis  
Fy 82 Funds: \$95,000

## CONTRACT RESEARCH SUMMARY

Title: Immunodiagnostic Markers for Breast Cancer

Principal Investigator:  
Performing Organization:  
City and State:

Dr. Rajender K. Chawla  
Emory University  
Atlanta, GA

Contract Number: N01-CB-84308  
Starting Date: 9/30/78

Expiration Date: 3/29/82

Goal: To evaluate urinary/plasma levels of EDC1, the principal component of cancer-related proteinuria, as an immunodiagnostic marker for breast cancer.

Approach: Pure EDC1 and inter- $\alpha$ trypsin inhibitor (IATI) and high titer anti-serum to EDC1 will be prepared. Standardized immunologic methods (e.g. radio-immunoassay (RIA) or immunoelectrophoretic techniques) will be developed to monitor EDC1 in urine/plasma of 1) normal healthy women; 2) women with metastatic breast cancer; 3) patients with non-neoplastic diseases; and 4) preoperative patients with a localized breast mass. A postoperative longitudinal study will monitor EDC1 levels of patients in group 4 with malignant lesions.

Progress: Urinary levels of EDC1, (a trypsin inhibitor,  $M_r$  27.5K, antigenically related to a normal plasma protein, inter- $\alpha$ trypsin inhibitor (IATI,  $M_r$  160K) were determined in the groups of patients mentioned above. The data indicate: 1) significant increase ( $p < .01$ ) in EDC1 excretion in metastatic breast cancer group as compared to normals and non-cancer patients ( $98.6 \pm 11$  vs  $8.4 \pm 2.2$  vs  $14.6 \pm 4$  mg/g creatinine respectively); 2) a decline in urinary EDC1 in pre-operative patients with malignant lesions who underwent surgery; 3) elevated proteinuria in preoperative patients with malignant lesions as compared to those with benign lesions ( $43.1 \pm 7.6$  vs  $21.5 \pm 3.4$  mg/g creatinine); 4) a low grade EDC1-proteinuria in non-cancer patients with rheumatoid arthritis and with certain infections ( $\sim 25$  mg/g creatinine). Serum levels of  $\alpha_1$ -proteinase inhibitor,  $\alpha_1$ -antichymotrypsin, and C1-inactivator (all acute phase proteins) in metastatic breast cancer patients were also found to be elevated (50 to 105% increase over normals:  $p < .01$ ),  $\alpha_2$ -macroglobulin levels were unchanged and IATI levels were depressed in heavy excretors of EDC1. Elevated levels of the abovementioned acute phase inhibitors have been reported in inflammation and infections. In our group of metastatic breast cancer patients, the bioactivity of  $\alpha_1$ -proteinase inhibitor, as measured by the serum trypsin inhibitory capacity, was 35 to 50% of the normal values ( $1.2 \pm 0.2$  units/ml vs  $2.1 \pm 0.3$  units for normals) in spite of increased immunoreactive level of this inhibitor. It suggests that in our patients most of the systemic  $\alpha_1$ -proteinase inhibitor may be biologically inert. Thus these studies have not only established urinary EDC1 as a viable tumor marker but they may have also identified a unique disturbance in the profile of plasma antiproteinase in breast cancer.

Project Officer: Dr. Bernice T. Radovich  
Program: Diagnosis  
FY 82 Funds: 0

## CONTRACT RESEARCH SUMMARY

Title: Characterization of HLA Antigens of Donors' Lymphocytes.

Principal Investigator: Dr. Rene J. Duquesnoy  
Performing Organization: Blood Center of Southeastern Wisconsin  
City and State: Milwaukee, WI

Contract Number: N01-CB-04337  
Starting Date: 6/2/82 Expiration Date: 6/1/83

Goal: To analyze as carefully as possible donors' cell surface histocompatibility antigens in order to subsequently analyze the relationship between those antigens and the ability of those donors' cells to mount appropriate immune responses.

Approach: Analysis of cell surface antigens is performed by two different detection systems; serology and cellular typing. The serologic analysis is performed using carefully screened alloantisera in assays of complement-dependent cytotoxicity. The cellular analysis had been performed by analyzing the proliferative responses to homozygous typing cells (HTC) typing but now is done by analyzing secondary restimulation of lymphocyte populations selectively immunized against alloantigens in primary response (PLT), particularly against antigens of the SB locus.

Progress: As of 6/24/82, approximately 120 samples have been typed in this contract year by serologic techniques for HLA-A, -B, -C, and -DR. The results this year have further refined our understanding of linkage disequilibrium in the population between SB alleles and alleles of other Ia loci (DR and MT). Furthermore, they have facilitated understanding of patterns of occurrence in the population of two variant forms of the Bw44 molecule, which promise to be useful probes for correlation between protein structure and control of cell surface expression of histocompatibility antigens. Family studies of four dermatitis herpetiformis patients, in progress at the last report, have now clarified the importance of SB2 as a marker of DR3 haplotypes relatively unlikely to carry the gene(s) which confer susceptibility to that disease. Extensive efforts were made at DR-typing a macrophage-like cell line to facilitate studies of antigen presentation.

Expertise in SB typing has been extended, along with the generation of SB-specific primed typing reagents. As part of the standardization of SB-typing, a reference panel of 9 lymphoblastoid cell lines was expanded, cryopreserved, and has been used for local standardization and shipped to 6 other laboratories.

Significance to Cancer Research: Evidence from animal models and from epidemiologic studies in humans suggest that host cellular immune responses are crucial in determining the outcome of neoplastic diseases. Cellular immune responses are under control by genes of the major histocompatibility complex (HLA in man). In order to therapeutically manipulate these cellular immune responses, we must first understand their normal operation and genetic control.

Project Officer: Dr. J. Stephen Shaw  
Program: Immunology Support  
Technical Review Group: Ad Hoc Review  
FY 82 Funds: \$100,091



# CONTRACT RESEARCH SUMMARY

Title: Maintain an Animal Holding Facility and Provide Attendant Research Services

Principal Investigator:	Ms. Leanne DeNenno
Performing Organization:	Cor Bel Laboratories, Inc.
City and State:	Rockville, MD

Contract Number:	N01-CB-04336	
Starting Date:	11/1/79	Expiration Date: 10/31/82

Goal: Maintain colonies of inbred mice (10,000 animals), inbred rats (500 animals), and rabbits (20 animals) and carry out selected breeding protocols with these animals as specified by the project officer. These animals are to be maintained in support of intramural research programs in the Immunology Branch, NCI.

Approach: Colonies of mice, rats, and rabbits are to be housed and fed according to National Research Council standards. Technical manipulations and breeding are to be carried out as directed by the project officer.

Progress: Performance on this contract has been highly satisfactory. The animal colonies have been established and are being maintained according to National Research Council standards. Animal health has, in general, been excellent, and breeding protocols have been satisfactory. Recordkeeping and transferring of animals to and from the NIH Campus have all been satisfactory.

Significance to Cancer Research: This animal colony is necessary in support of intramural research programs in the Immunology Branch of NCI. Many of these programs are concerned with the immune response to cancer.

Project Officer: Dr. David H. Sachs  
Program: Immunology Support  
Technical Review Group: Ad Hoc Review  
FY 82 Funds: \$313,400

CONTRACT RESEARCH SUMMARY

Title: Computer Services

Principal Investigator: Mr. Francis A. McDonough  
Name/Address: Department of the Treasury  
Performing 1435 G Street, N.W.  
Organization: Washington, D.C. 20220

Contract Number: Y01-CB-90316  
Starting Date: Nov. 15, 1979

Expiration Date: Sept. 30, 1982

Goal: To provide computer facility for mathematical computations related to biological systems modeling carried out in the Laboratory of Mathematical Biology and other groups in DCBD, NCI.

Approach: Facility is accessed through remote terminals.

Progress: This facility is used only in a backup capacity because our own VAX computer is taking over most of our computations.

Project Officer: Dr. Mones Berman  
Program: Cancer Biology Support  
Technical Review Group: Ad Hoc  
Relevance Review Group:  
FY '82 Funds: \$2,000.

Site Visit Date: N/A

## CONTRACT RESEARCH SUMMARY

Title: Radioimmunoassay and Enzyme Immunoassay of Immunoglobulin Molecules and Antibodies

Principal Investigator: Dr. Charles A. Bowles  
Performing Organization: Hazelton Laboratories, Inc.  
City and State: Vienna, Virginia

Contract Number: N01-CB1-4344  
Starting Date: 6/30/81      Expiration Date: 6/29/82

Goal: To perform radioimmunoassays of immunoglobulin molecules as well as ELISA assays of antibody molecules in lymphocyte culture supernatants or in biological fluids.

Approach: The contractor is to quantitate human IgG, IgA, IgM, IgE, lambda and kappa light chains in various fluids using double antibody radioimmunoassay procedures and reagents defined and supplied by the project officer. Furthermore, the contractor is to measure antibodies produced by lymphocytes stimulated by antigens in vitro. This contract provides critically required research support for the study of immunodeficiencies that are associated with a high incidence of malignancy and on the cause of the immunodeficiency associated with malignancies.

Progress: The contract has established radioimmunoassays for IgG, IgA, IgM and lambda and kappa light chains of man and ELISA assays for antibodies. These assays were used to quantitate immunoglobulin and antibody synthesis by human lymphocytes in in vitro cultures. Patients with the adult T cell leukemia associated with a retrovirus were shown to have a malignant expansion of a suppressor T cell whereas patients with the Sezary syndrome have an expansion of helper T cells. Patients with ataxia-telangiectasia and a high incidence of neoplasia were shown to have defects in helper T cells and of B cells that leads to a deficiency of IgA, IgE, and the IgG2 subclass. The assays for immunoglobulin molecules have been an integral part of studies of the arrangement and rearrangement of immunoglobulin genes that are required for a stem cell to mature into a B cell making an immunoglobulin molecule. Furthermore, these assays have been of value in categorizing malignancies such as the non-T, non-B cell leukemia of childhood that previously had been of controversial origin. These studies are defining the nature of disorders of the immune system related to cancer.

Significance to Cancer Research: These studies helped elucidate the abnormalities of the immune system associated with the development of cancer. They have assisted in the categorization of malignancies of the lymphoid system and have provided important new insights into the disorder of cell maturation of such malignancies.

Project Officer: Dr. Thomas A. Waldmann  
Program: Immunology Support  
Technical Review Group: Ad Hoc Review  
FY82 Funds: \$243,763

# CONTRACT RESEARCH SUMMARY

Title: Maintenance and Development of Inbred and Congenic Resistant Mouse Strains

Principal Investigator: Ms. Martha McGowan  
Performing Organization: Litton Bionetics, Inc.  
City and State: Kensington, MD

Contract Number: N01-CB-94325  
Starting Date: 2/1/79      Expiration Date: 1/31/82

Goal: To maintain a colony of inbred pedigreed strains of mice which are needed to support ongoing NCI intramural research in transplantation immunology.

Approach: The contractor maintains a colony of approximately 40 special inbred and congenic resistant strains of mice by pedigreed brother-sister mating. Quality control testing is carried out at each generation by cytotoxicity typing of animals from each strain. Alloantisera are raised between mouse strains to assist in this quality control typing, and sera and animals are shipped by the contractor to collaborating investigators at NIH and elsewhere.

Progress: The contractor has maintained all inbred and congenic resistant strains of mice in excellent condition. Breeding of each strain and of hybrid strains, recordkeeping, and quality control testing have all been highly satisfactory. A backcrossing program has been instituted for all congenic resistant strains in order to keep the backgrounds of these strains identical. This involves backcrossing of each congenic to the reference background line once every 6-10 generations. This program has also been very satisfactory to date. Five new recombinant H-2 haplotypes have been identified during the process of this backcrossing, and these have been bred to homozygosity and established as new valuable inbred congenic strains.

Hybridoma reagents have been produced, stored and shipped starting with cell lines developed by the Project Officer. Antisera for histocompatibility antigen typing have been prepared in a variety of combinations and have been found to be excellent reagents. A series of new strain-restricted typing sera have been produced in order to identify each strain in the colony and distinguish it from all other strains. Shipping of animals and sera to collaborating investigators at NIH and elsewhere has been very satisfactory. The animals shipped from these pedigreed colonies have generally been of excellent health and have provided breeding stock for the production of larger numbers of experimental animals in numerous laboratories.

Significance to Cancer Research: This animal facility is needed for the breeding and maintenance of these inbred congenic resistant strains of mice. These animals make possible research on individual histocompatibility antigens and, in particular, the role of the major histocompatibility complex in the transplantation of tissues and cells and in the immune response to cancer.

Project Officer: Dr. David H. Sachs  
Program: Immunology Support  
Technical Review Group: Ad Hoc Review  
FY 82 Funds: \$9,876.00

## CONTRACT RESEARCH SUMMARY

Title: Induction, Transplantation, and Preservation of Plasma Cell Tumors in Mice and the Maintenance of Special Strains

Principal Investigator: Martha J. McGowan  
Performing Organization: Litton Bionetics, Inc.  
City and State: Bethesda, MD

Contract Number: N01-CB-94326

Starting Date: 3/1/79

Expiration Date: 1/31/82

Goal: Transplantation, preservation induction, and shipping of plasmacytomas, T- and B-cell lymphomas in mice. Breeding of rare strains of mice, hybrids, and wild mice.

Approach: To maintain a closed conventional colony of BALB/c, BALB/c sublines, and BALB/c congenic strains for use in plasmacytoma induction and for the maintenance of a bank of transplantable plasmacytomas and other lymphocytic tumors. The mice in this environment are suitable for long-term plasmacytoma induction studies and for the generation of congenic strains of the BALB/c background. New BALB/c congenic lines, carrying genes and chromosomal segments from the plasmacytomagenesis-resistant DBA/2, C57BL, C3H and CBA backgrounds are being bred for use in plasmacytoma induction studies to identify genes involved with the genetic susceptibility of plasmacytomagenesis. A screening bank of myeloma proteins is maintained for the detection of new antigen binding myeloma. A wild mouse colony is maintained.

Progress: Over 400 shipments were made to NIH, foreign, and domestic investigators. Items shipped included ampoules of frozen viable tumor cells; pedigreed and wild mice and tumor products. Forty-eight (48) congenic strains are under development. In 5 of these a translocated chromosome is the marker and in another 11 a paracentric inversion is the marker. There were a total of 69 generation advances made in this period. Forty-seven (47) inbred strains were maintained by pedigree brother-sister mating. Four new strains were added to the colony. Thirty-one (31) wild mouse stocks were maintained including 13 new acquisitions. Eleven (wild x inbred) hybrid were bred.

Significance to Cancer Research: Provides essential support for the study of plasmacytomagenesis (carcinogenesis) with the specific goal of determining the genetic basis of susceptibility to tumor induction by mineral oil. Supplies essential biological material for investigators studying the biology of neoplastic plasma cells, tumor immunology, the genetics of immunoglobulins, and immunoglobulin synthesis.

Project Officer: Dr. Michael Potter  
Program: Tumor Immunology Support Program  
Technical Review Group: Immunology Support Contract (Ad Hoc Review)  
FY 82 Funds: \$ 0



## CONTRACT RESEARCH SUMMARY

Title: Maintenance and Development of Inbred and Congenic Resistant Mouse Strains

Principal Investigator (Administrative):	Ms. Martha McGowan
Co-Principal Investigator:	Mr. J. Scott Arn
Performing Organization:	Litton Bionetics, Inc.
City and State:	Kensington, MD

Contract Number: N01-CB-25585

Starting Date: 3/1/82

Expiration Date: 1/31/87

Goal: To maintain a colony of inbred pedigreed strains of mice which are needed to support ongoing NCI intramural research in transplantation immunology.

Approach: The contractor maintains a colony of approximately 40 special inbred and congenic resistant strains of mice by pedigreed brother-sister mating. Quality control testing is carried out at each generation by cytotoxicity typing of animals from each strain. Alloantisera are raised between mouse strains to assist in this quality control typing, and sera and animals are shipped by the contractor to collaborating investigators at NIH and elsewhere.

Progress: The contractor has maintained all inbred and congenic resistant strains of mice in excellent condition. Breeding of each strain and of hybrid strains, recordkeeping, and quality control testing have all been highly satisfactory. A backcrossing program has been instituted for all congenic resistant strains in order to keep the backgrounds of these strains identical. This involves backcrossing of each congenic to the reference background line once every 6-10 generations. This program has also been very satisfactory to date. Five new recombinant H-2 haplotypes have been identified during the process of this backcrossing, and these have been bred to homozygosity and established as new valuable inbred congenic strains.

Hybridoma reagents have been produced, stored and shipped starting with cell lines developed by the Project Officer. Antisera for histocompatibility antigen typing have been prepared in a variety of combinations and have been found to be excellent reagents. A series of new strain-restricted typing sera have been produced in order to identify each strain in the colony and distinguish it from all other strains. Shipping of animals and sera to collaborating investigators at NIH and elsewhere has been very satisfactory. The animals shipped from these pedigreed colonies have generally been of excellent health and have provided breeding stock for the production of larger numbers of experimental animals in numerous laboratories.

Significance to Cancer Research: This animal facility is needed for the breeding and maintenance of these inbred congenic resistant strains of mice. These animals make possible research on individual histocompatibility antigens and, in particular, the role of the major histocompatibility complex in the transplantation of tissues and cells and in the immune response to cancer.

Project Officer: Dr. David H. Sachs

Program: Immunology Support

Technical Review Group: Intramural Support Contract Proposal Review Committee

FY 82 Funds: \$438,854

## CONTRACT RESEARCH SUMMARY

Title: Induction, Transplantation, and Preservation of Plasma Cell Tumors in Mice and the Maintenance of Special Strains.

Principal Investigator: Martha J. McGowan, Judith Wax  
Lawrence D'Hoostelaere  
Performing Organization: Litton Bionetics, Inc.  
City and State: Bethesda, MD

Contract Number: N01-CB2-5584  
Starting Date: 02-01-82      Expiration Date: 1-31-87

Goal: Induction transplantation, preservation and shipping of plasmacytomas, T- and B-cell lymphomas in mice. Breeding of (congenic) strains of mice, to find genes controlling susceptibility and resistance to the induction of plasma cell tumors by pristane Maintenance of wild mouse colony.

Approach: Maintain a closed conventional colony of inbred and congenic strains of mice, suitable for maintaining mice for long term plasmacytoma induction experiments. Develop BALB/c congenic strains carrying plasmacytoma genesis resistance (PCT-R) genes. Carry out procedures for identifying markers used in the construction of congenic strains. Maintain colonies of pedigreed wild mice. Harvesting and shipment of N<sub>2</sub>-frozen transplantable tumors, serum, ascites, tissues, high molecular weight DNA, pedigreed breeders to qualified investigators and collaborators.

Progress: Excellent progress. 48 congenic strains are under development, 10 are being tested at N6 for susceptibility plasmacytomagenesis. Studies on the dose and interval of pristane to standardize and quantitate plasmacytomagenesis are near completion. Contractor continues to fill numerous request from highly qualified investigators for material not available elsewhere. Many of these studies with wild mice and congenic mice have revealed unexpected new polymorphisms of Mammary Tumor Proviruses; immune responses to phosphorylcholine, susceptibility to systemic and cutaneous Leishmaniasis, IgH, k, and λ, C and V-genes histone genes, Lymphocyte alloantigens.

Significance to Cancer Research: Provides essential support for the study of plasmacytomagenesis (carcinogenesis) with the specific goal of determining the genetic basis of susceptibility to tumor induction by mineral oil. Supplies essential biological material for investigators studying the biology of neoplastic plasma cells, tumor immunology, the genetics of immunoglobulins, and immunoglobulin synthesis.

Project Officer: Dr. Michael Potter  
Program: Immunology Support  
Technical Review Group: Intramural Support Contract Proposal Review  
Committee

FY 82 Funds: \$606,100

## CONTRACT RESEARCH SUMMARY

Title: Radioimmunoassay of Immunoglobulin Molecules

Principal Investigator: Dr. James Harness  
Performing Organization: Meloy Laboratories, Inc.  
City and State: Springfield, Virginia

Contract Number: N01-CB-63932

Starting Date: 3/14/81

Expiration Date: 3/13/82

Goal: To perform radioimmunoassays of immunoglobulin molecules IgM, IgA, IgD, and IgE in lymphocyte culture supernatants or in other biological fluids.

Approach: The contractor was to perform determinations of human IgG, IgA, IgM, IgD, IgE and kappa and lambda light chains and mouse IgA and IgM by double antibody radioimmunoassay by procedures defined by the project officer and using reagents supplied by the project officer. This contract provided critically required research support for studies on the nature of immunodeficiencies that are associated with a high incidence of malignancy and on the cause of the immunodeficiency associated with malignancies of the B cell or plasma cell system. In addition, these studies are directed at defining retained immunological capabilities of T cell leukemias.

Progress: The contract expired on 3/13/82. The contract has established radioimmunoassays for IgG, IgA, IgM, IgE and lambda and kappa light chains of man for IgA and IgM of the mouse. These assays were used to quantitate the rate of immunoglobulin synthesis by pokeweed mitogen stimulated, peripheral blood lymphocytes of man or of splenic lymphocytes of the mouse in in vitro cultures. Patients with a T cell leukemia associated with the Sezary syndrome have been shown to have a malignant expansion of helper T cells whereas a patient with acute lymphocytic leukemia was shown to have a malignancy of suppressor T cell precursors. A subset of patients with common variable hypogammaglobulinemia has been shown to have excessive numbers of suppressor T cells that inhibit gamma-globulin synthesis by B cells. Certain patients with post marrow transplant immunodeficiency have deficient B cell function whereas others have excessive suppressor T cell activity. Patients with multiple myeloma have hypogammaglobulinemia due in part to excessive numbers of suppressor macrophages. These studies are defining the nature of disorders of the immune system that led to a high incidence of cancer. In addition, they provide information on the cause of immunodeficiency that arises secondary to certain forms of malignancy. Finally, these studies have provided insights into the retained functions of T cell leukemias.

Significance to Cancer Research: These studies helped elucidate the status of the immune system in patients with a high incidence of cancer. In addition they aid in categorizing malignancies to the T cell or B cell system.

Project Officer: Thomas A. Waldmann, M.D.  
Program: Cancer Biology Support  
Technical Review Group: Ad Hoc Review Group  
FY 82 Funds: \$ 0

## CONTRACT RESEARCH SUMMARY

Title: Molecular Biologic Studies of Tumor Viruses

Principal Investigator: Dr. Richard S. Howk  
Name/Address: Meloy Laboratories  
Performing Organization: Rockville, MD

Contract Number: N01-CB-04342

Starting Date: 6-30-81

Expiration Date: 6-29-82

Goal: To provide support for studies of the structure and expression of tumor viruses.

Approach: Viral biochemical, serologic, and biological parameters are monitored in different systems to define the regulatory mechanisms of tumor virus expresession and to relate the expression to tumor production.

Progress: Mouse cells transformed by bovine papilloma virus (BPV) contain 5 different RNA transcripts. Each of the transcripts has the same 3° end, and each is contained within the 69% viral DNA fragment which was previously shown to contain the sequences required for transformation by BPV DNA. The sequences which encode the transforming gene(s) of BPV have been localized genetically within the 69% fragment of the viral DNA to a segment which is no larger than 2.3 kb. Other sequences within the 69% fragment control the expression of the transforming sequences and apparently determine if the BPV DNA will remain unintegrated.

Four different genes with sequences homologous to the p21 transforming gene of Harvey and Kirsten murine sarcoma viruses have been molecularly cloned from normal human cells. Two are closely related to the Harvey type gene and two are more closely related to the Kirsten viral p21 gene. The structure of one human Harvey type gene is very similar to that of a rat Harvey type gene. This normal human gene also has the capacity to induce oncogenic transformation of mouse cells when the p21 protein encoded by this gene is expressed at high levels by virtue of attaching a retroviral LTR to this gene in vitro.

A complete avian collagen gene has been molecularly cloned. The location of the exons of this gene, which are distributed over more than 50 kb, have been determined. The gene is being used to study the interaction between cellular transformation and collagen synthesis.

Significance for Cancer Research (NCP Objective 6 Approach 2)

Project Officer: Dr. Douglas R. Lowy  
Assistant Project Officer: Dr. Ira H. Pastan  
Program: Cancer Biology Support  
Technical Review Group: Ad Hoc Committee  
FY 82 Funds: \$273,570



## CONTRACT RESEARCH SUMMARY

Title: A Study of Phylogenetic Aspects of Neoplasia

Principal Investigator:  
Name/Address  
Performing Organization:

Dr. John C. Harshbarger  
The Smithsonian Institution  
Registry of Tumors in Lower Animals  
Washington, D.C.

Contract Number: N01-CB-33874

Starting Date: 7/1/73

Expiration Date: 9/30/82

**Goal:** To collect, examine, classify, and preserve neoplasms in cold-blooded vertebrate and invertebrate animals and to identify species and habitats where neoplasms appear at unusually high frequencies.

**Approach:** The principal investigator directs the operation of a registry of tumors in lower animals. Specimens are acquired from personal field investigations or through submittal by other investigators. The specimens are examined grossly, histologically, and in some cases by electron microscopy. Diagnoses are established and the specimens are described. The world literature on tumors in lower animals is collected. Field investigations and experimental inductions of tumors in lower animals are carried out. Publications of the findings are made. The Registry also serves in a consulting capacity to other agencies concerned with diseases in lower animals, such as the Environmental Protection Agency, the U.S. Bureau of Fisheries (Commerce Dept.), and the Environmental Health Center. A complete collection of the literature on neoplasms in ectothermic animals is maintained and kept current. Key information in the literature is abstracted and stored on computer tape and is available for retrieval. Data on specimen accessions is likewise stored in a computer system.

**Progress:** Specimen accessions during the past year numbered 238, bringing the total to 2609. The literature collection has added 190 papers, now totalling 3945. Data from these have been stored on computer tape and are retrievable by author, species, organ, diagnosis, and other key words. About half of the new accessions were confirmed to be neoplasms, while the remainder were parasitic, infectious, toxic, traumatic, or developmental diseases. Neoplasms were mostly from teleost fishes, amphibians, reptiles, and molluscs. In the invertebrate phyla, lesions that appear to be neoplasms by morphological criteria were found only in molluscs and arthropods (insects alone represented) with the exception of one parasitic trematode bearing a ganglioneuroblastoma. About 50 types of neoplasms have been recognized to occur at relatively high prevalences in certain species in certain locations. Types of tumors accessioned are listed in the 1981-1982 Progress Report of the Registry. Specimen contributions came from many foreign countries. Seven original reports were published by the PI during the 1981 fiscal year.

**Significance for Cancer Research:** Field studies and anatomical studies indicate environmental carcinogens that may be of importance in human cancer epidemiology or may be useful in designing analytical experiments to determine mechanisms of tumorigenesis. The continued apparent paucity of neoplasms among invertebrates other than insects and molluscs points to a need for basic research in this area.

**Project Officer:** Clyde J. Dawe, M.D. **Program:** Cancer Biology Support  
**Technical Review Group:** Ad Hoc Committee **FY 81 Funds:** 180,200  
**Site Visit Date:** No site visit



## CONTRACT RESEARCH SUMMARY

Title: Preparation of Purified Wheat Proteins and Wheat Protein Fractions

Principal Investigator:  
Performing Organization:  
City and State:

Dr. Donald Kasarda  
U.S. Department of Agriculture  
Berkeley, CA

Contract Number: Y01-CB-60312

Starting Date: 10/1/81

Expiration Date: 9/30/82

Goal: To obtain chemically defined fraction of wheat gliadin for use in studies of gluten-sensitive enteropathy (coeliac sprue).

Approach: Wheat gluten will be chemically fractionated and subjected to cyanogen bromide cleavable. Homogeneous fragments of gliadin, as well as gliadin itself, will then be supplied.

Progress: During the past year the contractor has supplied A-gliadin as well as chemically defined fragments of A-gliadin according to schedule. We have utilized these materials in studies of the immune response to toxic fraction of gliadin. In particular, we have stimulated interleukin-2 (IL-2) production in patients T cells using these materials. These on-going studies will be used to establish whether or not gluten-sensitive enteropathy is due to an Ir gene defect.

Significance to Cancer Research: Gluten-sensitive enteropathy is a disease associated with a high incidence of malignancy. Elucidation of the pathogenesis of gluten-sensitive enteropathy will provide insight into the factors which lead to the onset of malignant disease.

Project Officer: Dr. Warren Strober  
Program: Cancer Biology Support  
Technical Review Group: Breast Cancer Task Force Committee  
FY 81 Funds: \$25,000

# CONTRACT RESEARCH SUMMARY

Title: Interaction of Exercise, Dietary Carbohydrate and Cancer Cachexia in Rats

Principal Investigator:  
Performing Organization:  
City and State:

Dr. Richard A. Ahrens  
University of Maryland  
College Park, MD

Contract Number: N01-CB-94327  
Starting Date: 6/1/81

Expiration Date: 5/31/82

Goal: To investigate the separate and interactive effects of quantitatively imposed exercise and variation in dietary carbohydrate source on the systemic response of rats to growth of tumors.

Approach: The first phase of the project consisted of determining optimum ranges of exercise schedule and dietary carbohydrate source. The current phase consists of determining effects of imposed exercise and changes in carbohydrate source (oligo- or polysaccharides) and the interaction of these with tumor growth of food intake, body composition and N conservation.

Progress: Experimental work has been completed on effect of exercise and carbohydrate source and the interaction of these with growth of Walker 256 tumor on the variables under study. Analysis of final data on body composition is in progress. Imposed exercise depresses food intake, bodyweight, protein, minerals and lipid. Imposed exercise in the presence of tumor further reduces lipid but increases, relative to sedentary tumor-bearing, protein and minerals. Particularly, exercise reduced tumor-induced depletion of skeletal muscle protein. There is some indication that exercise may retard and even reverse tumor growth (in practice, this possible effect seriously complicated the experimental protocol and limited the data obtained). High starch substituted for high sucrose as carbohydrate source depressed tumor growth and slightly elevated N conservation of non-tumor-bearers. Generally the dietary effect was smaller than the exercise effect. Interaction of tumor, exercise and diet is complex and analysis has not yet been completed.

A preliminary report of some of this work has been presented to Am. Physiol. Soc., April, 1982 (Deuster and Ahrens, Fed. Proc. 41: 1752 (Abst. # 8667), 1982). Two scientific papers reporting the work and results in detail are in preparation. This contract terminates 31 May, 1982.

Significance to Cancer Research: Information concerning procedures that might retard or prevent the wasting, N depletion and hypophagic effects of cancer are of great potential importance to knowledge of origins and possible strategies for combating cachectic decay in cancer.

Project Officer: Ms. Susan Ficker  
Program: Cancer Biology Support  
Technical Review Group: Ad Hoc Committee  
FY 82 Funds: 0









NATIONAL CANCER INSTITUTE

ANNUAL REPORT

October 1, 1981 through September 30, 1982

TABLE OF CONTENTS

Page

DIVISION OF CANCER BIOLOGY AND DIAGNOSIS

DIRECTOR

XIII

Summary Report

Office of the Director

Project Report:

CB-08900 Lymphocyte Cell Surface Antigens, Normal and Neoplastic 1

Laboratory of Biochemistry

Summary Report 7

Project Reports:

CB-00333 Biochemical Basis for Defective Differentiation in Granulocytic Leukemia 19

CB-00366 Biosynthesis and Assembly of Intracellular Components 25

CB-00375 Homogeneity and Structure of Proteins 29

CB-00945 Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells 33

CB-05202 Isolation, Fractionation, and Characterization of Native Nucleoproteins 40

CB-05203 Immunochemical Purification and Characterization of Immunocytes and Components 46

CB-05210 Cellular Controls over Growth and Inducible Processes 49

CB-05214 DNA Synthesis in Mammalian Cells 55

CB-05231 Role of Subunit Interactions in Enzyme Chemistry and Cellular Regulation 60

CB-05234 Interrelations Between the Genomes of SV 40 and African Green Monkeys 66

CB-05244 Organization of Repeated DNA Sequences in African Green Monkeys 70

CB-05251	Structure and Transcription of the Ribosomal Genes in <u>Drosophila melanogaster</u>	75
CB-05252	Sequence Organization in the Genome <u>Drosophila melanogaster</u>	78
CB-05254	Gene Expression in <u>Xenopus laevis</u>	81
CB-05258	Molecular Studies of Eukaryotic Gene Regulation	84
CB-05261	Manifestations of Human Cancer	88
CB-05262	Eukaryotic Gene Regulation and Gene Transfer	92
CB-05263	Eukaryotic Chromatin Structure and Gene Regulation	96
CB-05264	Characterization of a Mouse Repetitive Gene Family	99
CB-08701	Regulation of Cellular and Viral Gene Expression	101

#### Laboratory of Molecular Biology

Summary Report	107
----------------	-----

#### Project Reports:

CB-08000	Regulation of Gene Activity by Cyclic Nucleotides and Transforming Proteins	112
CB-08001	Role of Cyclic and Transforming Viruses in the Regulation of Cell Behavior	115
CB-08006	Control of Gene Expression in Bacteriophage Lambda	118
CB-08010	The Morphologic Mechanisms of Motility and Neoplastic Transformation in Culture	121
CB-08011	Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein	125
CB-08700	Structure and Expression of Collagen Genes; Regulation of Genes in Bacteria	130
CB-08702	Endocytosis in the Thyroid Gland	136
CB-08703	Thyroid Hormone Synthesis and Storage	138
CB-08704	Thyroid Growth and Involution	140
CB-08705	Genetic and Biochemical Analysis of Cell Behavior	143
CB-08706	Mammary Carcinogenesis: MMTV - Cell Interaction	147
CB-08707	Relationship of Genetic and Non-Genetic Factors in Mouse Mammary Tumorigenesis	151

CB-08709	NAD Metabolism and ADPriboseylation of Proteins	154
CB-08710	DNA Replication <u>In Vitro</u>	157
CB-08711	Mechanism of Site-Specific Recombination in Bacteriophage Lambda	159
CB-08712	The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior	161
CB-08714	Mode of Action of a Bacterial Function Involved in Cell Growth Control	165
CB-08715	Control of Synthesis of a Transformation-Dependent Secreted Glycoprotein	168
CB-08717	Role of Carbohydrates in Processing and Stabilization of Glycoproteins	171
CB-08718	Genetic Interaction between <u>E. Coli</u> K12 and Bacteriophage $\lambda$	175
CB-08719	Development and Uses of Eukaryotic Vectors	178
CB-08750	Regulation of Regulatory Proteins in <u>E Coli</u>	186
CB-08751	Genetic Regulatory Mechanisms in <u>Escherichia Coli</u> and its Bacteriophage	190
CB-08752	Mechanism of the Transport of Thyroid Hormones into Animal Cells	194

#### Laboratory of Pathophysiology

Summary Report	197
----------------	-----

#### Project Reports:

CB-00306	Modification of Amino Acids in Search of Possible Anti-tumor Agents	205
CB-00941	Genetic and Other Factors Affecting Marrow Transplantation in Irradiated Mice	209
CB-00942	Effects of Gamma-Irradiation on Nucleic Acids and Proteins	214
CB-00944	Total Metabolism of Cancer Cachexia	221
CB-05211	Poly(ADP-ribose) and Chromatin Structure and Function	224
CB-05216	Cyclic Nucleotide-Growth Regulatory Mechanism	227
CB-05219	In Vitro Simulation of Hormone-Dependent Mammary Tumor Regression	232

CB-08205	Application of Artificial Capillary Culture Technique to Hormone Production	237
CB-08210	Control of Lymphocyte Protein Synthesis and Response to Biological Agents	240
CB-08212	From Gene to Protein: Structure, Function and Control in Eukaryotic Cells	246
CB-08215	Isolation and Characterization of the Angiogenesis Factor	250
CB-08217	Early Changes in mRNA Metabolism in Regressing MTW9 Tumors	252
CB-08218	Hormonal Influences on Whey Milk Protein Gene Expression in Rat Mammary Explants	254
CB-08219	cDNA Sequence of $\alpha$ -LA, Whey Phosphoprotein & k-Protein: Their Genomic Methylation	257
CB-08220	Structural Gene Organization of Rat $\alpha$ -LA and its Genomic DNA Sequence	261
CB-08225	Membrane Structure and Dynamics of Normal and Neoplastic Mammary Gland	263
CB-08226	Hormones and Growth Factors in Development of Mammary Gland and Tumorigenesis	265
CB-08229	Role of Dietary Lipids in Mammary Cancer	271
CB-08230	Hormonal Control of Mammalian Follicular Maturation and Oogenesis	274
CB-08235	Membranes of Human Normal, Thrombasthenic and Bernard-Soulier Platelets	278
CB-08247	Basement Membrane Collagen Degradation and Its Role in Basement Membrane Physiology	280
CB-08249	Hormonal Control of Growth of Normal and Neoplastic Mammary Cells	283
CB-08250	Fracture-Label: Cytochemical Labelling of Freeze-Fractured Membranes	286
CB-08251	Growth Factor Production by Neoplastic Rat Mammary Epithelial Cells	289
CB-08252	Interaction of Growth Factors & Tumor Promoters in Cell Growth & Differentiation	293
CB-08256	Role of Hormones and Cyclic Nucleotides in Mediating Cell Growth and Differentiation	297

CB-08257	Tumor Cell Invasion of Native Connective Tissue (human amnion) <u>in vitro</u>	301
CB-08258	Tumor Desmoplasia: Characterization of the Collagens Produced by Human Tumors	305
CB-08259	Interactions of Metastatic Tumor Cells with Extracellular Matrix	308
CB-08263	Essential Fatty Acids and Mammary Gland Development and Tumorigenesis	312
CB-08264	Cyclic Nucleotide and Carcinogenesis	315
CB-08265	Isolation and Characterization of a Type V Collagenolytic Enzyme	318
CB-08266	Structure and Biological Function of Basement Molecules	321
CB-08268	Structure, Topology, and Dynamics of Tight Junctions	324
CB-08269	Membrane Differentiation: Role of Integral Components in Membrane Domains	328
CB-08270	Intracellular Compartmentalization of Membrane Glycoproteins	331
CB-08271	T Lymphocyte Heterogeneity: Labelling of Lectin Receptors of Transmembrane Proteins	335
CB-08272	Membrane Glycoproteins and Glycolipids of Normal and Transformed Human Cells	338
CB-08273	The Biochemistry of Invasiveness	340
CB-08274	Regulation of Lactogenic Hormone Receptors in Mammary Tissue	343
CB-08275	Asymmetric Topology of Glycolipids in Membranes of <u>Acanthamoeba Castellani</u>	349
CB-08276	Role of Calcium in Mediating Cell Growth and Differentiation	351
CB-08277	Antibodies as Probes of Cyclic Nucleotide Function in Human Breast Cancer Cells	354
CB-08278	Migration of Capillary Endothelium by Angiogenesis Effectors	358
CB-08279	Effect of Proline Analogs on Normal and Neoplastic Breast Epithelium	360



## Laboratory of Mathematical Biology

Summary Report	363
----------------	-----

### Project Reports:

CB-08300	SAAM, Modeling and Applications	366
CB-08303	Movement of Molecules in Membranes	368
CB-08306	Kinetic Modeling of Human Plasma Lipoprotein Metabolism	372
CB-08320	Macromolecular Conformations	375
CB-08323	Assay Quantitation	378
CB-08331	Glucose-Insulin Kinetics. Modeling of Carbohydrate Metabolism	381
CB-08335	"Targeting" Liposomes for Selective Interaction with Specific Cells and Tissues	383
CB-08340	Physical Chemistry of Antibody Effector Function	387
CB-08341	Physical Chemical Studies of Lipid-Protein Interactions	390
CB-08342	Theory of Receptor-ligand Biophysics	393
CB-08348	Receptor-ligand Kinetics and Function	396
CB-08349	Characterization of Macromolecular Flexibility	398
CB-08353	Perpetuation and Eradication of Viruses in Large Populations	400
CB-08354	Enzyme-Substrate Complexes	402
CB-08357	Cell Interactions	405
CB-08358	Epidemic Cycles	407
CB-08359	Monoclonal Antibodies <u>In Vivo</u> for Diagnosis and Therapy of Tumors	409
CB-08360	Calculation of the Three-Dimensional Structures of Membrane-Proteins	411

## Laboratory of Pathology

Summary Report	415
----------------	-----

### Project Reports:

CB-00853	Surgical Pathology and Postmortem Section	432
----------	---	-----

CB-00852	Exfoliative Cytology Applied to Human Diagnostic Problems and Research Problems	437
CB-00518	Fate of IgE Bound to Mast Cells	442
CB-00520	Surface Disposition of Cell Bound IgE with Time	444
CB-00530	Analysis of Bile from Cases of Gallbladder Cancer	446
CB-00545	Extracellular Matrix Synthesis by Human Tumors <u>in Vitro</u>	448
CB-00594	Pathology of Family Cancer Studies	450
CB-00884	Ultrastructural Organization of Basal Lamina	452
CB-00508	Immune Response of CBA/N Mice to Oligosaccharides Coupled to Protein Carriers	454
CB-00510	Glucose-containing Tetrasaccharide in Human Urine	457
CB-00511	Carbohydrate Heterogeneity in Alpha Subunits of Human Polypeptide Hormones	460
CB-00523	Complex Carbohydrate Released from Mammalian Cells by Trifluoroacetyolysis	462
CB-00525	Analysis of Oligosaccharides by Combined Gas Chromatography Mass Spectrometry	465
CB-00529	Structure of the Carbohydrate Portion of Human Haptoglobin	468
CB-00549	Hybridoma Antibodies to Oligosaccharide Haptens	471
CB-00521	Epithelial-Mesenchymal Interactions in Neoplastic Development	474
CB-00538	Identity of X Cells in "Tumors" of Fishes	482
CB-00533	Study of Virus Cell Interaction and Biological Characterization of Human Tumors	485
CB-00542	Studies on Oncogenic Primate DNA Viruses	487
CB-00543	Characterization of the Papillomaviruses	490
CB-00547	The Use of Papillomavirus DNAs as Eukaryotic Cloning Vectors	493
CB-00546	Specific Incorporation of a Fluorescent Probe by Transformed Lymphocytes	496
CB-00548	Monoaminergic Receptors on Lymphocytes Visualized by FACS Analysis	497

CB-00550	Immunologic Characterization of Malignant Lymphomas	499
CB-00551	Stimulation of Phagocytosis by a Peripheral T-Cell	501
CB-00552	Malignant Lymphomas: Analysis with Monoclonal Antibodies	503
CB-00553	Control of Fibrinogen Gene Expression	505
CB-00880	Malignant Lymphomas and Hypercalcemia: Analysis with Antibody Against OAF	507
CB-00881	Regulation of Cell Growth by Transferrin Receptors	509
CB-00882	Use of the Cell Sorter to Clone Hybridoma Cells Secreting Idiotype	511
CB-00883	Malignant Lymphomas: Analysis with Monoclonal Antibodies on Tissue Sections	513
CB-00517	Report from the Pathological Technology Section	515
CB-00885	Automated 2D Gel Analysis Using Computerized Data Base Methods	517
CB-00886	Analysis and Synthesis of Nucleic Acid Secondary Structure	519

#### Metabolism Branch

Summary Report	523
----------------	-----

#### Project Reports:

CB-04002	Defects in Immunoregulatory Cell Interactions in Patients with Immune Dysfunction	539
CB-04003	Studies of Porphyrin Metabolism in the Tumor-Bearing Host and Porphyria	550
CB-04004	Regulatory Functions of Amino Acids	553
CB-04014	Studies of the Immune Response in Normal and Pathological States	559
CB-04015	Development and Function of Humoral and Cellular Immune Host Defense Mechanisms	569
CB-04016	Mechanism of the Anabolic Action of Growth Hormone	579
CB-04017	Biology of the Immune Response	584
CB-04018	Study of Human Immune Defense Mechanisms and Its Control	597
CB-04019	Immunoregulatory Cells in Human Neoplasia	602
CB-04020	Genetic Control of the Immune Response to Natural Antigens	609

## Dermatology Branch

Summary Report 621

### Project Reports:

CB-03657	Immunopathologic Mechanisms Involved in Inflammatory Skin Disease	628
CB-03666	Detection and Analysis of Circulating Immune Complexes	633
CB-03659	Therapy of Skin Cancer, Disorders of Keratinization, and Cystic Acne	637
CB-03630	Effects of Vitamin A and Analogs on Chick, Mouse and Human Skin	647
CB-03638	Studies of DNA Repair in Human Degenerative Diseases	650
CB-03650	Biochemical Characterization of Mammalian Melanosomes	655
CB-03649	Enzymatic Control Mechanisms in Melanogenesis	659
CB-03654	Applications of SEM to Soft Biological Tissues	662
CB-03663	Tumor Virus Expression <u>In Vitro</u> and <u>In Vivo</u>	667
CB-03656	Chemistry, Structure and Biosynthesis of Mammalian Epidermal Keratin	675

## Immunology Branch

Summary Report 683

### Project Reports:

CB-05003	Cell-Mediated Cytotoxicity	687
CB-05018	Membrane Damage by Immune Mechanisms	692
CB-05021	Antigens Determined by the Murine Major Histocompatibility Locus	695
CB-05023	Transplantation Antigens of Swine	701
CB-05033	Immunotherapy of Human Cancer	706
CB-05035	Characterization of the Lymphocyte Receptor for IgG (FcγR)	709
CB-05036	Genetic Control of the Immune Response to Staphylococcal Nuclease	713
CB-05038	Cell-Mediated Immunity to Hapten Modified Syngeneic Lymphocytes in Mice	717

CB-05050	Studies on the Structure and Function of the Constant Portions of Immunoglobulins	721
CB-05055	Regulatory Influences on Cell-Mediated Immune Responses	731
CB-05058	Immunoregulation of Antibody Synthesis and Secretion	733
CB-05062	Application of Rapid Flow Microfluorometry to Cell Biology	736
CB-05064	Genetic Control of the Immune Response In Vitro	743
CB-05065	Immunologic Function of Accessory Cells	746
CB-05067	Genetic Control of Human In Vitro Cellular Immune Responses	749
CB-05069	Expression of Ia Antigens on Functional Cell Subpopulations	752
CB-05083	Genome Organization of Murine Major Histocompatibility Complex	755
CB-05085	Development of Syngeneic Tumor Immunity	758
CB-05086	Immune Response Gene Regulation of the Immune Response In Vitro	761
CB-05087	T Cell Recognition in the Mixed Lymphocyte Response	764
CB-05088	Effects of Graft vs. Host Reactions on Cell-Mediated Immunity	767
CB-05090	Role of Accessory Cells in B Cell Activation	770
CB-05091	Target Antigen Recognition by Cytotoxic T Lymphocytes	772
CB-05093	Environmental Influences on Self-Tolerance	775
CB-05094	Role of the Thymus in Generation of the Self-MHC Specific T Cell Repertoire	778
CB-05095	Regulation of Cell-Mediated Immunity by Germ Cells	781
CB-05096	Identification and Function of Intracellular Calcium-Containing Organelles	784
CB-05098	Non-H-2-linked Genetic Control of Cell-Mediated Cytotoxic Responses	787
CB-05099	Immunogenetic Effects of Murine Cytomegalovirus on Induced and Natural Immunity	790
CB-05100	The Role of HLA Genes in Human Disease	793
CB-05101	Definition of Gene Products of the Human Major Histocompatibility Complex	796



CB-05102	Morphologic Studies of Cellular Interactions in the Immune Response	801
CB-05103	Structure and Function of Cloned Lymphocytes	806
CB-05104	Detection and Analysis of H-2 Variant Cell Lines from Murine T Cell Lymphomas	808
CB-05105	Specificity of Human Cytotoxic Effector Cells Generated by Stimulation with Concanavalin A	810
CB-05106	Analysis of the T Cell Alloreactive Repertoire	812
CB-05107	T Cell Responses to Minor Histocompatibility Antigens	816

#### Macromolecular Biology Section

Summary Report	819
----------------	-----

#### Project Reports:

CB-05097	Structure of Thyroglobulin and Interaction with Membrane Receptors	821
CB-05545	Expression of Cellular Antigens in Transformed Cells and in Embryonic Cells	825
CB-05526	Cell Surface Changes in Spontaneously or SV40 Transformed Mouse Cell Lines	831
CB-05546	A Common Protein in Embryonic Differentiation and in Cell Transformation	836
CB-05544	Cell Surface Changes in Transformed Mouse Cell Lines	840

#### Laboratory of Immunobiology

Summary Report	845
----------------	-----

#### Project Reports:

CB-08525	Immunotherapy of Primary Autochthonous Cancer	853
CB-08527	Immunotherapy of Animals with Clinical Stage II Malignant Disease	855
CB-08528	Mechanism of Delayed Hypersensitivity and Tumor Graft Rejection	858
CB-08530	Mechanisms of Immune Eradication of Tumors	864
CB-08550	Modification of Tumor Cells and Immune Cytolysis	868
CB-08551	Induction of Tumor Immunity by Chemotherapy	874

CB-08552	Mechanism of Complement Fixation and Action	877
CB-08557	Immunoassay of Fluid-Phase and Cell-Bound Antibodies and Antigens	880
CB-08575	Inflammation	883
CB-08576	Immunological Mechanisms of Tumor Rejection	887
<u>Laboratory of Cell Biology</u>		
	Summary Report	893
<u>Project Reports:</u>		
CB-03200	Factors Influencing the Induction, Growth and Repression of Neoplasms	902
CB-03229	Structure and Cloning of Histocompatibility and Tumor Antigens	907
CB-04833	Biological Studies of Various Normal, Virus-Infected and Malignant Cells	912
CB-04834	Genetic Mechanisms of Carcinogenesis and Biological Modifier as a Defense Mechanism	916
CB-05550	The Role of Retroviruses in Oncogenesis and Tumor Immunogenesis	922
CB-05552	Mammalian Cellular Genetics and Cell Culture	927
CB-05553	Immunoglobulin Structure and Diversity. Characterization of Cell Membrane Proteins	936
CB-05596	Pathogenesis of Plasma Cell Neoplasia: Characterization of Antigen-Binding Proteins	940
CB-08726	Biological and Biochemical Characterization of Transplantation Antigens	948
CB-08727	Organization and Control of Material in Immunoglobulin-Secreting Plasmacytomas	951
CB-32555	Immune Response to Tumor Associated Antigens	955

DIVISION OF CANCER BIOLOGY AND DIAGNOSIS  
NATIONAL CANCER INSTITUTE

SUMMARY REPORT OF THE DIRECTOR

October 1, 1981 through September 30, 1982

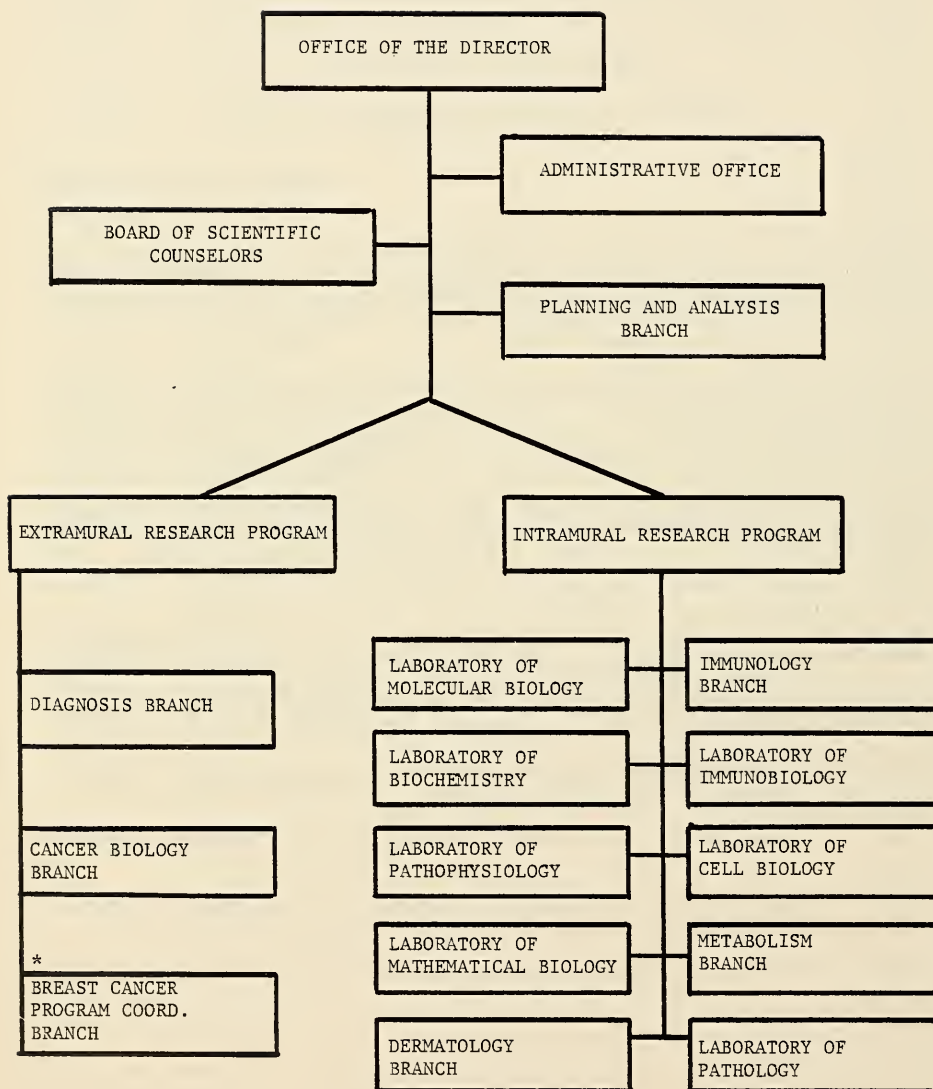
INTRODUCTION

The Division of Cancer Biology and Diagnosis consists of both intramural and extramural research programs in the biology and diagnosis of cancer. The Division also coordinates the activities of the Breast Cancer Task Force and the Lung Cancer Screening Program. A close coordination is maintained with other divisions of NCI in areas of common interest.

The intramural research program consists of three broad areas: cancer biology, immunology and clinical research. The laboratories performing basic research in cancer biology include the Laboratories of Biochemistry, Molecular Biology, Mathematical Biology and Pathophysiology. The Immunology Program includes work in the Immunology Branch and the Laboratories of Immunobiology and Cell Biology. Three clinical branches work in the fields of Dermatology, Metabolism and Pathology. A fourth organizational element, under the Associate Director for Extramural Research Program, manages the four major extramural programs: Tumor Biology, Immunology, Cancer Diagnosis, and Breast Cancer Task Force.

During the reporting period the Immunodiagnosis Lab was transferred to the Division of Cancer Treatment; while a part of DCBD throughout most of the year, Breast Cancer Program Coordinating Branch was transferred to DRCCA to join other organ site programs. Finally, all of the radiological projects in the diagnosis program were transferred to DCT, where a new radiology program was initiated. The current organizational chart is reflected on Page XIV.

The report of the Extramural Program is given in the second volume, which includes both contract and grant funded research.



\* NOTE: Transferred to DRCCA

## Cancer Biology

This report will include three program areas: Tumor Biology (Laboratories of Biochemistry, Molecular Biology, Pathophysiology and Mathematical Biology), Clinical Research (Laboratory of Pathology, Dermatology Branch and Metabolism Branch) and Immunology (Immunology Branch, the Macromolecular Biology Section, and the Laboratories of Immunobiology and Cell Biology).

### Laboratory of Biochemistry

A major research activity of the Laboratory of Biochemistry (Dr. M. Singer, Chief) has been the study of biochemical and cellular actions of hormones. Investigators have studied the regulation of growth hormone and prolactin production in a rat pituitary adenoma cell line (GH<sub>3</sub>) and in hybrids between GH<sub>3</sub> and a mouse cell line (LB82) which does not express either gene. The hybrids are likewise non-expressing. cDNA probes for both rat growth hormone (GH) and prolactin (Prl) were used to isolate the corresponding genes. Further analysis showed that rat and mouse GH and Prl genes are in the hybrid cells, and that little or no GH RNA was produced in the hybrids; thus the dominant (mouse) L cell regulation probably acts by preventing rat GH gene transcription. Preliminary results suggest that the loss of prolactin synthesis in the hybrids occurs by a similar mechanism. Portions of the GH gene have been cloned into two vector systems and used to transform cells. The transformants will be examined for hormone-controlled GH expression. Additional studies have been directed towards examining the phenylpyrazole glucocorticoid lysis of leukemic cells, which are resistant to the standard glucocorticoids. Its toxicity in mice, its effect on several tumor cell lines, and binding of the <sup>3</sup>H-labeled compound to cells are being investigated.

The regulation of collagen biosynthesis in normal and transformed cells continues to be a focal topic of research activity in the Section of Biosynthesis. The role of ascorbic acid in collagen metabolism is being reinvestigated using the scorbutic guinea pig model. Under conditions of acute ascorbic acid deficiency, collagen synthesis in the excised calvarial bone is decreased relative to total protein synthesis. This effect appears to be unrelated to the role of ascorbate as cofactor in proline hydroxylation. Pair-feeding experiments indicate that the decrease in collagen synthesis in bones from scorbutic animals is secondary to decreased food intake and a negative nutritional balance rather than a direct effect of ascorbate deprivation on collagen metabolism.

Investigations of the enzymes and accessory proteins involved in DNA synthesis continue. Evaluation of the structure-function relationships of *E. coli* DNA polymerase I large fragment suggested that a single lysine residue in an essential dNTP-binding site is one target for pyridoxal-5-phosphate (PLP) modification. Studies have been undertaken to localize this lysine residue in the primary sequence of the enzyme.

Another group has concentrated on studying a family of endogenous retroviral genes related to the mouse intracisternal type-A particle (IAP). Multiple individual IAP genes have been cloned from a BALB/c gene library and characterized. Recently this work has been extended to the related genes in other mouse species in rats (350 copies), and in Syrian hamster (800 copies). The analysis of cloned genes and whole genomic DNA indicates that amplification began between



2.5 and 4.5 million years ago in the lineage of Mus musculus but was probably a relatively recent occurrence in the Syrian hamster line of descent. The rat IAP gene family shows a great deal of internal heterogeneity, suggesting an evolutionally more distant amplification event. The data suggest that the IAP gene family has undergone independent amplification events at different times in the evolution of the species studied.

Additional work is directed towards examining the localization of IAP on specific mouse chromosomes and possible proximities between IAP sequences and other known genes. Thus far, multiple IAP gene copies have been definitively associated with chromosomes 6, 15, and X in M. musculus. IAP sequence elements have been found near several (but not all) immunoglobulin light chain variable region genes ( $\kappa$  and  $\lambda$ ) and near at least one other endogenous retrovirus-like (VL30) DNA sequence.

Recombinant DNA techniques have been used to develop a plasmid, phage, and bacterial vector system which allows the isolation, characterization, and comparison of prokaryotic transcriptional regulatory signals. The system has been used to introduce and functionally characterize point mutations in several promoter and terminator signals. The effects on signal efficiency have been monitored and related to the structural alteration of the site. Another vector system has been developed which has the potential of producing essentially any protein within the bacterial cell. This system has been used successfully to overproduce a 97 amino acid polypeptide (cII) encoded by phage  $\lambda$ , which serves as a positive activator of transcription by RNA polymerase. Interactions of this protein with itself, with DNA, and with RNA polymerase are being studied.

Previous work demonstrated that under the appropriate conditions prokaryotic genes could be expressed efficiently in eukaryotic cell-free systems. High levels of the bacterial galK are produced in a variety of mammalian cells transfected with an SV40-plasmid recombinant vector carrying the E. coli galK gene. The ability to obtain expression of an easily assayable gene product from a structurally defined fusion vector has now permitted studies of functional complementation by the bacterial enzyme of primary cells isolated from human patients with galK-deficiency galactosemia or other galK-deficient mammalian cells, as well as studies of transcriptional and translational regulation in mammalian cells. These vectors have been modified so as to contain a second independent, selectable, assayable marker (the xanthine-guanine phosphoribosyl-transferase gene product) which permits quantitative analysis of various eukaryotic regulatory signals. Thus, recombinant DNA techniques allow examination of regulatory sequences, overproduction of useful gene products, and analysis of the effects of naturally occurring mutations.

The structure and expression of rRNA genes from Drosophila continue to be studied with the primary aim of understanding the cause for the inactivity of interrupted genes. Run-off transcription in isolated nuclei of Drosophila cells showed that insertions are not transcribed in vitro; suggesting that isolated nuclei preserve in vivo control in this respect. Interrupted rRNA genes appear to be transcribed up to the point of insertion but not beyond. Plasmids containing modified rDNA are being constructed that will allow the assay of transcription of homologous rDNA introduced into cells. The organization of the genome of Drosophila melanogaster has been studied concentrating on the structure of a transposable DNA sequence called the F element. Three DNA regions containing F elements were isolated from one stock of D. melanogaster. Corresponding regions

from another stock of D. melanogaster did not contain any F element. F elements appear to constitute a novel class of transposable sequences in Drosophila. A female sterile mutant called fs(1)h that leads to homeotic transformations in Drosophila is being studied with the aim of isolating the DNA encoding this gene.

Gene expression during development in Xenopus laevis has been studied in several ways. A cDNA library highly enriched for sequences expressed in gastrula but rare or absent in unfertilized eggs has been prepared to enable the study of genes expressed early in development. A cDNA clone derived from mRNA for calmodulin has been isolated and sequenced. The size of major calmodulin genes has been determined. Using defined, cloned double-stranded cDNA probes for various genes expressed in differentiated chick muscle cell cultures, the corresponding genomic sequences for  $\alpha$  and  $\beta$  actin, vimentin, a major intermediate filament protein, and glyceraldehyde phosphate dehydrogenase have been isolated.

Another research program has been concerned with the mechanism of the coordinated regulation of cellular processes by  $\text{Ca}^{2+}$  and cAMP. In most, if not all eukaryotic cells, the action of  $\text{Ca}^{2+}$  is modulated by the  $\text{Ca}^{2+}$ -receptor protein, calmodulin. Two proteins, phosphorylase kinase and calcineurin, exhibit dual  $\text{Ca}^{2+}$  regulation: calmodulin in the case of phosphorylase kinase, calcineurin B in the case of calcineurin. Each interacts with exogenous calmodulin as well. Determination of the amino acid sequence of calcineurin B is almost completed. This protein is clearly homologous to calmodulin. Calmodulin cannot interact simultaneously with more than one target protein; only one calmodulin fragment peptide 78-148, has preserved both its  $\text{Ca}^{2+}$  binding property and its ability to interact with phosphorylase kinase and cyclic nucleotide phosphodiesterase. Experimental data suggest that different enzymes may recognize the same calmodulin domain but have different requirements for activation. The calmodulin binding proteins become activated upon limited proteolysis and lose their  $\text{Ca}^{2+}$  dependence. All of them are therefore composed of two domains, a catalytic domain and a regulatory, binding domain. Current work is directed towards the characterization and isolation of the interacting sites on calmodulin and its target proteins. These studies will lead to a better understanding of the ability of calmodulin to interact with so many different proteins.

Another research interest in the laboratory concerns the relation between the genomes of simian virus 40 (SV40) and the African Green Monkey (AGM). A library of the AGM genome in  $\lambda$  bacteriophage yielded three different AGM DNA segments that are homologous, in part, to the region around the SV40 origin of replication. Each homologous segment is a few hundred base pairs in length. Experiments with two of the AGM segments are being carried out using SV40-based vectors. Experimental data indicate that the monkey DNA segments do not replace the SV40 control region in support of DNA replication. They do, however, provide transcriptional start sites at which the synthesis of messenger RNA begins. The monkey segments do not contain all the elements necessary for transcription. A special region of the SV40 genome known as the "72 base pair repeat" is by itself insufficient for transcription. It must, however, be included at some position in the vector. The position and orientation is irrelevant. These results suggest that the "72 base pair repeat" enhances transcription. The starting sites within the monkey sequences are being mapped by the S1 and primer extension methods in order to study the specific sites.

Considerable research effort has been aimed at understanding the dynamic nature of the primate genome. The highly repeated sequences in the genome of the AGM

were chosen for study. A new monkey satellite with a ten base pair repeat length was discovered and the cloned portions determined. This "deca-satellite" is highly polymorphic. Three short DNA segments that occur hundreds of thousands of times interspersed in the monkey genome are also being studied. Evidence was obtained to support the theory that one of these, the primate element called Alu, is a movable element. The other two elements were discovered in this laboratory during the past year. One of them is flanked by a duplication of the target site suggesting that it is movable. Both newly discovered elements hybridize with cellular RNA.

Analysis of somatic cell hybrids segregating human chromosomes was used to localize human *onc* gene analogues, immunoglobulin genes, and Ig pseudogenes to specific human chromosomes. Several large series of independent hybrid cell lines were isolated and the human chromosome content determined by isoenzyme analyses and karyotyping. DNA was isolated from each cell line and analyzed. Studies resulted in assignment of constant region kappa ( $C_K$ ) and lambda ( $C_\lambda$ ) Ig genes.

Using highly purified populations of  $\text{Lyt2}^+$  and  $\text{Lyt2}^-$  T cells it was found that help by  $\text{Lyt2}$  helper cells for cytotoxic effector cell (CTL) generation in mixed lymphocyte culture is not antigen-specific. The amount of CTL activity that can be generated is not limited by the number of CTL precursors but by the amount of help that is provided by the  $\text{Lyt2}^-$  helper cells. Other recent experiments have shown that macrophage-like cells are required for such help. The data suggest that the  $\text{Lyt2}$  helper cells provide lymphokine IL2. These studies should provide a better understanding of the differentiation of CTL from precursors in the thymus. A further research goal is to better understand the molecular reactions between immune cells in T cell subpopulations and antigens bound to target cell surfaces that lead to *in vivo* cellular immune phenomena such as graft rejection and the graft-versus-host reaction.

Additional studies are aimed at identifying the regulatory factors that control the induction of synthesis of specific proteins associated with the differentiation of bone marrow leukocytes. A major goal of this research has been attained with the development of a rapid direct assay for granulocyte differentiation proteins. Another major goal has been achieved in the development of a cell culture system that permits the study of the regulation of the maturation of normal and leukemic immature granulocytes at the morphological and biochemical levels simultaneously. This has been accomplished by the selection of culture conditions that permit granulocyte differentiation to be studied in the absence of cell proliferation.

The transplantable, granulocytic leukemia (GL-13-BC) developed in inbred strain 13 guinea pigs is an attractive model for human chronic myelocytic leukemia (CML). The model is presently being used to develop new therapeutic regimens for delaying or presenting the onset of blast crisis, a stage of the disease that is almost always fatal in cases of human CML. Additional work continues on developing a system for grading human tumors by utilizing biological characteristics of tumor cells. Tumor cell attachment to new substrates is being compared to normal cell attachment using reflection contrast microscopy. A major characteristic of cancer cells, release from density dependent growth control, is also being studied as is the role of lamellar cytoplasm in tumor cell growth.



## Laboratory of Molecular Biology

The research effort of the Laboratory of Molecular Biology, (Dr. I. Pastan, Chief) is directed towards understanding the factors that control gene expression in animal cells and bacterial cells, and on using this information to define the biochemical basis for the abnormal growth and behavior of cancer cells. To understand how Rous sarcoma virus transformation alters cell growth and gene expression, the transforming protein from Rous sarcoma virus induced tumors was purified to near homogeneity. Using this purified enzyme, it has been shown that the phosphorylation of vinculin, a physiological substrate for the enzyme, is greatly stimulated by the presence of membrane phospholipids, indicating that the association of the enzyme with the plasma membrane is necessary to direct the activity of the enzyme towards specific substances. The tyrosine-specific protein kinase can also phosphorylate glycerol, raising the possibility that the enzyme may phosphorylate other intracellular substrates.

The activity of some collagen genes is under the control of Rous sarcoma virus transformation. The  $\alpha 2$  Type I collagen gene and the Type III collagen gene were isolated. Structural comparisons of the two genes indicate that they probably evolved from a common ancestral gene at least 200 million years ago. Further studies showed that the collagen gene is different from other genes previously studied in its regulation, indicating that malignant transformation may regulate gene activity in a unique manner.

To study regulation of gene activity, new plasmid expression vectors have been created which combine elements of the plasmid PBR322 and the virus SV40 with portions of cellular genes. The bacterial chloramphenicol acetylase gene has been inserted downstream from promoters of genes of interest. The enzyme chloramphenicol acetylase is not a normal product of animal cells, and is easily assayed. Recent experiments have shown that the avian sarcoma virus promoter is the most effective promoter yet found for expression of chloramphenicol acetylase in recipient cells after gene transfer. This may explain why other related avian tumor viruses can cause B cell lymphomas when inserted at the proper locus in chickens. Using such a vector, the collagen promoter has been inserted adjacent to the chloramphenicol acetylase gene, and the expression of the collagen promoter when introduced into animal cells is being studied. The 72 base pair repeat of SV40 has been found to be a very effective enhancer of collagen expression. The search for enhancing sequences within or close to the collagen gene continues. Other investigators have developed a cell-free system to investigate collagen gene expression, and have achieved successful transcription of collagen DNA using extracts of Rous sarcoma virus transformed chicken cells. This was the first time that host gene has been transcribed in a homologous in vitro transcription system.

Genetic and biochemical studies of the regulation of the growth and shape of Chinese hamster ovary (CHO) fibroblasts have continued. Mutants in CHO cells located in both the genes for  $\alpha$  and  $\beta$  tubulin genes have been isolated and have been found to affect cell growth by affecting mitotic spindle assembly. Gene transfer techniques have been developed that enable the transfer of genes into CHO cells. The successful development of these methods is of great importance because CHO cells are the most frequently used cell type for genetic studies and previously have been resistant to the transfer of genes.

Considerable research has been directed toward investigating the role of the plasma membrane in receiving signals from hormones, growth factors, the extracellular matrix and other cells, and on determining how these signals are transmitted to the genetic apparatus. Past work has shown that the steps in ligand internalization involve finding the cell surface receptors, clustering of receptor-ligand complexes in clathrin-coated pits and formation of a receptosome from a coated pit. Entry of molecules through this pathway allows them to enter the cell without being directly routed to lysosomes and rapidly destroyed. Some ligands such as epidermal growth factor and  $\alpha_2$ -macroglobulin are carried by the receptosome from the cell surface into the Golgi region where they become concentrated in coated pits of the Golgi and then transferred to lysosomes. Some ligands, such as vesicular stomatitis virus and adenovirus escape from the receptosome into the cytoplasm.

The biochemical processes involved in ligand internalization are not known. It has been found that proton ionophores inhibit endocytosis. The presence of sodium and bicarbonate in the medium are required for efficient endocytosis and do not interfere either with ligand binding or ligand clustering in coated pits. These treatments may slow or prevent the formation of the receptosome from coated pits, suggesting that the neck of the coated pit transiently closes and some type of an ion pump increases the isosmotic pressure within the "cryptic" coated pit producing a pressure sufficient to generate the receptosome.

Cancer cells are characterized by deranged control of synthesis of some proteins and of cell division, frequently a result of viral take-over of specific host machinery. The Laboratory of Molecular Biology also conducts basic research to understand the control mechanisms of protein synthesis, cell division and host-virus interactions using prokaryotic cells as model systems. The modulation of the inducible synthesis of galactose metabolizing enzymes is being studied. To characterize the complete regulatory features of the galactose operon, investigators have now identified the in vivo transcription initiation sites corresponding to the two promoters ( $P_{G1}$  and  $P_{G2}$ ). A novel in vivo DNA binding assay of the galactose repressor molecules has been developed which should be applicable to other systems. Investigators have isolated a new set of galactose operator mutants ( $O^s$ ), one of which is located within the first structural gene. This is the first demonstration of a functional operator located inside a structural gene.

Bacteriophage  $\lambda$  when expressed from a prophage causes several changes in host cell physiology. One of these is the expression of neighboring host genes due to extension of transcription from the phage promoters into these genes caused by overcoming the normal transcription termination signals in genes by phage anti-termination factor N. Several proteins participate in cellular transcription reactions, eg., Rho, L, NusB and ribosomal proteins L11 and S10. Studies continue to determine how these proteins interact with each other and with phage N protein to effect transcription termination and anti-termination, and have already shown that NusB and L11 specifically interact with L protein for anti-termination purposes.

Several regulatory genes are known in E. coli whose products regulate a range of other functions, including cell division. A change in the level of these molecules or mutation in their genes have pleiotropic effects. To understand how these molecules act and how their levels are controlled, investigators have cloned their corresponding genes to characterize the products, and are studying



their regulation in vivo by fusing their promoters to the easily assayable  $\beta$ -galactosidase structured gene, and in vitro by using purified DNA and transcription proteins.

### Laboratory of Pathophysiology

The research program of the Laboratory of Pathophysiology, (Dr. P. Gullino, Chief) is focused on pathophysiology of the mammary gland and its tumors, the biochemical changes related to onset and cessation of lymphocyte proliferation and the structure and function of biological membranes.

Studies on the milk protein gene expression during normal differentiation of mammary cells have been initiated in an attempt to understand the underlying mechanism involved in the malignant transformation of mammary epithelial cells. The role of cAMP in the regulation of mammary tumor growth has been pursued. Studies show that the cAMP receptor level was consistently enhanced during tumor regression while the receptor level for estrogen was consistently decreased, and the in vitro synthesis of several polypeptides instructed by poly(A)<sup>+</sup> RNA of the tumor was consistently changed. The changes did not occur when hormone independent tumors were treated in a similar way. The response observed in vivo could be reproduced in vitro on tumor slices. The antagonistic action between cAMP and estrogen at the nuclear level suggests a therapeutic potential for cAMP in human breast cancer.

In an attempt to understand the role of dietary lipids in the development of breast cancer, the types, quantities, and sources of fatty acids taken up by mammary epithelium have been analyzed. Studies indicate that prolactin stimulates histamine, which activates the release of free fatty acids from the proximal mammary adipocytes. The prolactin primed epithelium then selectively takes up the unsaturated fatty acids, part of which become localized in the membrane phospholipids. The fat cells of the gland provide an effective buffer which normally restricts the availability of unsaturated fatty acids to the epithelium. Exceeding the buffering capacity, which may occur in individuals on high fat diets, may sensitize mammary epithelium to basal levels of circulating hormones, and thereby increase the proliferation rate of the epithelium and expand the population at risk to malignant transformation.

Alterations of the hormone-receptors on or within cells modify the response of target tissues to various hormones, and thus control cellular growth or function. Studies have shown that the number of detectable prolactin receptors is controlled in vivo by the circulating levels of prolactin or growth hormone by a positive feedback mechanism. Inhibition of in vivo prostaglandin (PG) synthesis results in a loss of existing prolactin receptors (PRL) and prevents their induction by PRL. As membrane fluidity increases the number of PRL receptors also increases. The data show that PRL regulates its own receptor by modifying target membrane fluidity. This may occur through modification of prostaglandin. Utilizing a newly developed assay, it has been demonstrated that regressing tumors have an increased capacity to bind PG, and that copper increases this binding capacity.

The effects of metastasizing cells on the structure and biological function of the molecules in the basement membrane have been examined. Chemical digestion of laminin revealed two distinct subunits, each of which mediates different biological functions. Recent data indicate that the tumor cell surface has a

receptor for laminin. Degradation of laminin may facilitate the passage of immune cells and tumor cells through the basement membrane.

Previous work revealed that type IV and type V collagens are degraded by specific collagenases. Type IV collagenase has been purified and its cleavage products have been partially characterized. Secretion of this collagenase is directly related to the metastasizing capacity of neoplastic cells. A neutral metal protease has been identified and partially purified which specifically cleaves native type V collagen. The enzyme is secreted in culture by malignant macrophages. The presence of a Type V collagenase in tumor cells may enhance their ability to invade other tissues.

The mechanism of the angiogenic event in tumor growth is being studied with the purpose of developing an assay that can detect acquisition of angiogenic capacity in biopsies, and therefore have possibilities of predicting high risk of neoplasia; this also would help in finding a way of interfering with tumor growth by interfering with the neoformation of vessels. Copper appears to be an indispensable component of the angiogenesis process. Experimental data showed that three different molecules were all able to induce angiogenesis, only if they were bound to copper.

Studies of the regulation of protein synthesis during lymphocyte proliferation, and the response of lymphocytes to biologically active substances continue to be emphasized. Eight specific proteins (I-peptides) are induced by purified interferon in human lymphocytes. The relative amount of these proteins varies among breast cancer patients. These differences may be used to evaluate the effectiveness of interferon on patients with mammary carcinoma. A cDNA sequence coding for human immune interferon has been identified. The DNA sequence codes for a polypeptide which has properties characteristic of  $\gamma$ -interferon. Treatment with various preparations of interferon increased production of type IV collagenase and greatly enhanced the invasiveness of the Ewings sarcoma cells.

Newly developed "fracture-label" techniques have been used to examine the distribution and partition of glycoproteins in the intracellular membrane of secretory and non-secretory molecules. These techniques avoid the problem of cross-examination of membrane fractions, making it possible to ascribe precise locations to glycosylated products. As the distribution and topology of the membranes of normal cells is characterized, it may be possible to identify topochemical changes that occur during malignant transformation.

#### Laboratory of Mathematical Biology

The research activities of the Laboratory of Mathematical Biology, (Dr. M. Berman, Chief) focus on studies of membrane biophysics, immunology, macromolecular configurations, kinetics of metabolic systems and computational and modeling methodologies. Studies of the insertion and organization of molecules (proteins, lipids) in membranes, and the cell biology and pharmacology of lipid vesicles are of primary interest.

Work has begun on the in vivo use of monoclonal antibodies for diagnosis and treatment of tumors. The initial aim has been to delineate pharmacokinetic principles in well-defined, reproducible systems. The results are being used to design optimal agents for diagnostic gamma camera imaging and, possibly for therapy.

The development and application of mathematical techniques for describing receptor clustering on the plasma membrane has continued. The results were used to propose a theory of the response of sensitized basophils and mast cells in an attempt to explain the biochemical pathway selected by the cell (histamine release, specific desensitization, or non-specific desensitization).

Experimental applications of the theory of cell surface events were extended to IgG complexes interacting with Fc receptors on macrophages. This work is relevant to immune complex effector mechanisms, and may also provide insight into the analysis of data on a number of other systems.

Research within the laboratory also encompasses biological macromolecules and their properties. Stabilities of macromolecular conformations are determined by interatomic interactions. Short and medium range interactions appear to determine locations of regular  $\alpha$ -helices. Several simple models of long range intramolecular interactions have been formulated to facilitate investigations of protein folding pathways.

More detailed calculations of conformational energies, requiring evaluation of all interatomic distances, are feasible for protein fragments or small proteins. Such calculations have lead to postulation of new binding sites for lysozyme. Experimentally, it was found that the substrate displaced the site specific monoclonal antibody, thereby verifying the calculated binding sites. Similar calculations are being applied to the variable regions of the antigen combining site of myeloma immunoglobulins. Membrane embedded proteins are being studied, in particular their preferred conformations within the membrane. It has been found that mellitin and the leader peptide sequences are probably in the  $\alpha$ -helix conformation within membranes, in agreement with experimental data.

#### Laboratory of Pathology

The Laboratory of Pathology (under recently appointed Acting Chief, Dr. L. Liotta) is responsible for the services of surgical pathology and autopsy for the Clinical Center of the NIH. In addition, diagnostic electron microscopy studies and cytopathologic services, including exfoliative and fine needle aspiration are provided. The Laboratory of Pathology also provides all types of histological services and staining procedures for NCI scientists.

Research programs in various areas of experimental pathology are being carried out. An extensive study of the disposition of the IgE Fc receptor in the basophil membrane, employing monomeric ferritin-labelled IgE and basophil membrane ghosts, has been completed. No evidence for a cytoplasmic protrusion of the Fc receptor was found in reversed membrane vesicles, suggesting that an intramembranous process must be responsible for binding and crosslinking of IgE, as in the allergic response. There seems to be no direct link between the receptor and a cytoplasmic mediator of degranulation. Related work has demonstrated that only cross-linked IgE with its associated receptors is internalized. Both IgE and its receptor are degraded, exhibiting no surface re-expression of either.

In a recent study on cystinosis, it was shown that heterozygous cystinotics though clinically normal, have a diminished capacity to lysosomally process cystine. Such impaired lysosomes are detectable in peripheral blood neutrophils. This may allow detection of suspected or unknown carriers.



Studies of matrix synthesis by Ewing's tumor have clearly established that this tumor is a sarcoma in a very primitive state since it synthesizes type IV collagen, or "epithelial" collagen. These results suggest that this tumor is undifferentiated. Similar studies of "round-cell" tumors of childhood (neuroblastoma, primitive soft tissue sarcoma, and lymphoma) revealed unique profiles of matrix protein synthesis which can be distinguished from one another. This derivative approach to the detection of matrix protein synthesis by these tumors in vivo may be diagnostically useful.

Research continues on immunochemistry of complex carbohydrates. Current approaches include 1) determination of carbohydrate structures of glycoproteins and analysis of oligosaccharide mixtures; 2) development of hybridoma antibodies against oligosaccharide haptens, and 3) studies on the origin, metabolism, and excretion of a urinary oligosaccharide derived from glycogen. Preliminary data suggest that elevated excretion of this oligosaccharide in patients with rhabdomyosarcoma, Ewing's sarcoma, ALL, and some other tumors might be a useful indicator of tumor regression or recurrence during therapy.

Genetic and epigenetic factors in the mouse polyoma virus tumor system continue to be investigated. Molecular genetic analyses have been done on 5 cloned lines of polyoma virus. One line PY (LID), causes a high mortality (over 90%) in the 30-day period after birth, and high tumor incidence in survivors. The other, PY (A<sub>3</sub>), causes only a low mortality in the perinatal period and very few tumors in survivors followed by 9 months. Restriction enzyme mapping and base pair sequence analysis revealed that a single difference between Py (LID) and Py (A<sub>3</sub>) exists in a short non-coding segment of the Py genome. This segment from Py (LID) has been inserted into Py (A<sub>3</sub>), and the recombinant virus to determine whether it has biologic properties characteristic of Py.

Previous work demonstrated that certain lesions simulating neoplasms in marine fishes are composed largely of cells of protistan parasitic nature ("X cells"). Such lesions include the pseudobranch "tumors". In a recent study, involvement of thymus by X cells was found in a quarter of the fish with pseudobranch lesions. X cells have a tropism for skin, gill, and thymus. Developmentally, these sites are of ectodermal and pharyngeal endodermal origin, as are the organs of mice in which epithelial tumors are induced by polyoma virus. Thus a protistan parasite and a small DNA virus each possess a mechanism to stimulate mitogenically a group of cell types that have a common embryologic and phyletic derivation.

Other studies have focused on investigation of the mechanisms by which animal viruses interact with cells susceptible to lytic productive infection as well as the non-productive malignant transformation of cells. Human epidermal cells have been transformed with the cloned DNA fragments containing the entire early regions of the wild-type SV40 and of a tsA mutant of SV40 (tsA209). No transformants could be obtained using the cloned DNA of a tsA288 virus which is similar to tsA209, except for an additional deletion which renders it incapable of making a small t antigen. This suggests that a functional small t antigen as well as a functional large T antigen are required for transformation of primary cells. These transformants have altered growth properties which were shown to be due to a functional A gene product. The SV40 transformants quantitatively made less keratins than their non-transformed counterparts, and a lower percentage of the cells could be induced to form cross-linked envelopes by an ionophore. It is the expression of the A gene product which interferes with the expression of these differentiated properties in epidermal cells.

Additional work has focused on transformation with the human and bovine papillomaviruses. It was demonstrated that the BPV-1 genome remains exclusively extrachromosomal in transformed mouse cells. The viral transcripts present in BPV-transformed cells, as well as those present in productive fibropapillomas of cattle have been mapped. All the transcripts are derived from a single strand. The sequence of the bovine papillomavirus has been completed and only one strand has open-reading frames. There are two additional transcripts present in productively-infected cells which are not present in the transformed cells. The bodies of these messages are complementary to sequences in the non-transforming portion of the BPV-1 genome. One of these messages encodes the major capsid protein by DNA filter selection of the mRNA from a fibropapilloma and translates this into VP-1 in vitro.

Studies continue to examine the expression of selective markers cloned into a variety of transcriptional units in bovine papillomavirus. It was demonstrated that two selective markers (malignant transformation and ability to grow under selective media conditions) are closely linked. Analysis of the DNA showed that the hybrid DNA is present extrachromosomally but that a high degree of rearrangements have occurred. This selective marker, when cloned into a BPV-pML2 hybrid, is able to transform cells at high efficiency. The complete bovine papillomavirus genome, is highly efficient at transforming mouse cells and can be used as a shuttle vector for transporting DNA's between animal cells and prokaryotic bacteria.

The human B-interferon gene was inserted into a bovine papillomavirus vector. Mouse cells selected for their malignant phenotype were shown to synthesize a low amount of human beta interferon. The messenger RNA in the induced cells is coming from the authentic interferon promoter. While the complete BPV genome cloned into pML2 is able to transform mouse cells at high efficiency, the pML2 sequences are inhibitory to the transformation of mouse cells by the 69T fragment when they are covalently linked. A number of eukaryotic segments have been identified which provide a facilitation function when covalently linked to a BPV<sub>69T</sub>-pML2 hybrid DNA--the rat preproinsulin gene, the human growth hormone gene and the rat intergenic segment.

Several collaborative studies of the immunologic, cytochemical, and functional aspects of malignant lymphoma are underway. An extensive study evaluating the clinical importance of immunologic phenotype in patients with diffuse, aggressive non-Hodgkin's lymphomas has been recently completed. It has demonstrated that regardless of immunotype, patients have a similar remission rate and survival with aggressive chemotherapy.

Peripheral T-cell lymphomas have been the basis of several collaborative investigations. Previous studies suggested that the hypercalcemia which occurs with peripheral T-cell lymphomas may be due to the production of osteoblast-activating factor (OAF) by the neoplastic cells. Further studies in progress include the use of a monoclonal antibody directed against OAF to analyze neoplastic cells directly for this important biologic material. Hypercalcemia appears to be a particularly prevalent complication in patients with HTLV-associated T-cell malignancies and the production of OAF is being investigated in HTLV-positive lymphoid lines. A syndrome which simulates malignant histiocytosis is often seen in certain patients with peripheral T-cell lymphomas. It has been postulated that this syndrome might be due to the production of a lymphokine by the neoplastic T cells which could stimulate the phagocytic cells of the reticuloendo-



thelial system. Recent studies demonstrated that both normal and neoplastic T cells can elaborate factors which induce phagocytic activity in U937 cells, a cell line of true histiocytic origin. The factor produced by these cells can act independently of an effect on Fc receptors. A similar factor was identified in supernatants from T cells stimulated by concanavalin A, in which an increase in phagocytosis was seen independent of an effect on Fc receptors.

Using a battery of monoclonal antibodies and the fluorescence-activated cell sorter, the expression of various antigens has been investigated in non-Hodgkin's lymphomas. It has been demonstrated that lymphoblastic lymphomas are heterogeneous and include cases of T-, pre-B, and pre-pre-B origin. The lymphoblastic lymphomas with a T-cell phenotype reflect different stages in normal intrathymic differentiation, particularly late thymic differentiation, in contrast to T-ALL, which normally corresponds to an earlier stage of T-cell differentiation. The thymocyte antigen, OKT6, is present in less than half of cases of T cell lymphoblastic lymphoma. B-cell lymphomas can be subclassified based on expression of different antigens. Malignant lymphomas of intermediate lymphocytic differentiation have a unique phenotype and express both LEU1 and the ALL antigen (J5). These studies can be extremely useful in the staging of patients with non-Hodgkin's lymphomas. Additional studies have investigated the role of the transferrin receptor and growth of normal and neoplastic cells. Studies have shown that expression of OKT9 correlates with growth. Investigations are underway to correlate the expression of the transferrin receptor with the receptor for T-cell growth factor as identified by TAC binding.

Another project of interest has been the development of a model to study gene expression controlled developmentally and hormonally. The small family of genes which code for the three polypeptide chains of fibrinogen has been chosen for the study. To date, work has concentrated on the regulation of fibrinogen mRNA levels as well as the molecular cloning and structure of the rat and human fibrinogen genes. cDNA clones were obtained for each of the three chains of fibrinogen. Clones corresponding to fibrinogen chains were identified from the in vivo translation products of mRNA selected by cloned DNA's. The identification of each clone was confirmed by determining the nucleic acid sequence and aligning the predicted amino acid sequence with the known human amino acid sequence. It was found that the gamma chain of fibrinogen is encoded by two mRNAs which arise from a single gene by alternate splicing patterns. Two gamma chain proteins are produced which differ in length by 8 amino acids and have different carboxy termini. These two fibrinogen gamma chains are present in a wide variety of species including man and each chain is incorporated into fibrin polymers during coagulation. The structure of the rat fibrinogen genes has been studied by Southern blotting and by examination of genomic DNA clones for the three fibrinogen chains. It was found that each of the fibrinogen chains is encoded by a single gene per cell and that the alpha and gamma chains are tightly linked within 12 kb of each other.

Image processing and other computer techniques provide support for a variety of biologic studies ranging from the characterization of proteins which are associated with various directions of stem cell differentiation to methods of characterizing the secondary structure of nucleic acid molecules of known base sequence. The GELLAB system was developed to aid in the analysis of 2 dimensional electrophoretic gel analysis. Several collaborative biomedical projects involve the use of GELLAB to follow cell differentiation. In one system, GELLAB is applied to analyze changes in the levels of messenger RNAs associated with

differentiation along the separate pathways. An analysis of difference in malarial proteins synthesized by Plasmodium knowlesi parasites of different variant antigen phenotype uses GELLAB to characterize the proteins associated with or even perhaps definitive of the phenotypic variants. Success in this direction would represent a major step along the road to further understanding some of the complex immune responses in malarial infection.

Considerable effort has been expended on developing a system which can generate secondary structure, given the sequence of bases that make up a molecule. The combinatoric complexity of computing secondary structure from sequence data, employing thermodynamic data available on various base pairs and sequences, is a problem of continuing concern. Improvements in the core program now allow molecules of 420 bases to be run. The algorithm has been further developed so that much larger molecules can be run using a disk paging technique. Molecules of 1500 or more bases now are possible. An additional molecular drawing program was developed which eliminates the problem of overlap of complex structures which were topologically inherent in the older algorithm. Now the output of the secondary structure predicting programs can be viewed without the intervening interactive untangling process.

#### Metabolism Branch

The Metabolism Branch (directed by Dr. T. Waldmann) carries out clinical investigations aimed at: 1) determining the nature of immune processes that are of importance in the surveillance against neoplastic cells, 2) defining the nature of defects in the immunological system that result in an increased incidence of neoplasia, and 3) examining the physiological and biochemical effects that a tumor produces on the metabolism of the host.

A primary research goal has been to define the major events of cellular differentiation, cellular interaction and cellular biosynthesis involved in the specific circulating immune response. Special emphasis has been placed on defining the defects of B cell maturation and of regulatory T cell and macrophage interaction with B lymphocytes and plasma cells that that occur in patients with immunodeficiency diseases associated with a high incidence of malignancy, in patients with autoimmune disorders, as well as in patients with malignancies of the T or B lymphocyte systems.

As a stem cell matures into a B cell, rearrangement of the genes coding for immunoglobulin molecules occurs. Clones of human constant, joining, diversity and variable region genes have been used as probes to study the gene arrangements in T cell, B cell and non-T/non-B forms of human lymphocytic leukemia. These studies showed that lambda genes appear to remain in the germ line configuration in kappa producing B cells, whereas kappa genes are rearranged or deleted in lambda producing B cells. In contrast each of the T cell leukemias and lines studied displayed germ line patterns of both kappa and lambda light chain genes and in 90% of the cases, had germ line heavy chain constant, joining and diversity chain genes as well. Cells from patients with non-T/non-B acute lymphocytic leukemia, which consists of cells at early stages of development, were also examined. A series of categories of gene rearrangements were present in these cells that had not been seen in mature B or T cells. The patterns of immunoglobulin gene rearrangements in pre-B cells as well as B cell leukemias and lines suggests an ordered hierarchy of gene rearrangements that occurs as a stem cell matures into a B cell with mu genes preceding light chains, and kappa light chain genes generally preceding lambda.

Previous studies showed that the maturation of B cells into antibody producing plasma cells is carefully regulated by distinct subpopulations of lymphoid cells (helper T cells and macrophages). Recent data suggest that a separate population of suppressor T cells, may act as negative regulators of B cell maturation, inhibiting this process. Inactive thymus-derived prosuppressor cells require an interaction with another T cell (a suppressor inducer or activator) to become an active suppressor cell. To study these events, a series of techniques has been developed to examine the terminal differentiation of B cells into immunoglobulin synthesizing and secreting cells, the helper T cell function, and to detect both increased and decreased functional activities of suppressor effector T cells and their precursors and activators.

A major accomplishment of this laboratory has been the development of a culture and assay system which can quantitate the production of antibody which is generally restricted and antigen specific. It has been shown that this antigen specific response requires adherent cells and two distinct helper T lymphocyte populations. A helper T cell defect, as well as their intrinsic B cell defect, was demonstrated in cells from patients with ataxiatelangiectasia (A-T) utilizing this assay.

An enzyme linked immunoabsorbent assay (ELISA) was developed to examine specific antibody productions by peripheral blood mononuclear cells in vitro. Studies have been undertaken to define the T-cell subset(s) required for specific antibody production in vitro. It was found that OKT4<sup>+</sup>OKT8<sup>-</sup> T-cells but not OKT4<sup>+</sup> T-cells provided "help" for specific anti-influenza virus antibody production in vitro. Thus, there are two helper T-cell subsets, one of which can provide help for both CTL generation and antibody production in vitro and one which provides help for CTL generation but not antibody production. This assay was also used to evaluate the T-cell and B-cell defects involved in patients with various immunodeficiency diseases. Another area of major research activity has been directed towards defining the nature of the cellular structures involved in self/self and self/non-self recognition, and intercellular communication in immune and non-immune host defense processes. Previous studies identified a system of recognition of foreign target cells by non-immune mononuclear phagocytes based on the interaction of effector cell surface lectin-like receptors with carbohydrate determinants on the target cells. This recognition system can be found in primitive invertebrates, in mammals, and in man. Recent experiments have explored the possibility that such lectin carbohydrate interactions might also be involved in immunoregulatory signals between lymphoid cells. T cells of patients with infectious mononucleosis are profoundly inhibitory to the process of B cell activation in vitro by mitogens or by the Epstein-Bar Virus (EBV). D-mannose and methyl D-mannoside almost totally reversed the suppression caused by the infectious mononucleosis T cell permitting immunoglobulin production to occur in these cultures. In preliminary studies, a variety of synthetic mannose analogs have been tested and several compounds show activity at concentrations as low as 50  $\mu$ m. These and other studies appear to support the hypothesis that the sugar-lectin recognition has been conserved in evolution, and forms the basis of certain immunoregulatory cellular interactions as well.

The Epstein Barr Virus (EBV) is responsible for infectious mononucleosis, is associated with African Burkitt's lymphoma and nasopharyngeal carcinoma, and now appears to be involved in with Rheumatoid arthritis (RA). Suppressor T cell activity specific for EBV stimulated B cells, which is found in normal EBV immune subjects, was absent in the adult RA patients, even though they were immune to



EBV. This suppressor defect was restricted to responses induced by EBV, and was present in every case studied. In addition, all those patients with juvenile rheumatoid arthritis (JRA) who had multiple joint involvement have been EBV seropositive and have demonstrated the defect in EBV specific suppressor T cell activity seen in adult RA patients. These data suggest that the EBV may play an important but still poorly understood role in RA in adults and in a definable subgroup of JRA patients.

The techniques for assessing helper T cell activity were applied to homogenous populations of T cells from patients with T cell leukemias. Those patients whose T cells retained the capacity to help normal B cells in pokeweed mitogen stimulated cultures, had high IgA and IgE levels. In addition, the Sezary cell leukemias were associated with a circulating monoclonal immunoglobulin. Studies of Sezary leukemia cells confirm previous conclusions that Sezary cells are relatively mature T cells that are dedicated to helper interactions with B cells.

Extensive studies have been carried out on the leukemia associated with the human T cell leukemia lymphoma virus (HTLV) described by Gallo. Work in this laboratory helped to determine that the retroviral genome is present in certain T cell subsets, but not B cells from affected patients. These cells do not function as helper T cells, but act as suppressors of in vitro immunoglobulin synthesis, and appear to have an active interleukin II or T cell growth factor receptor. The data suggest that these cells are derived from a population of suppressor T cells, and are distinct from the Sezary leukemias both in terms of surface phenotype and function. Two other cells have been identified within the suppressor T cell series. A variety of positive and negative immunoregulatory molecules, including suppressive factors, are being examined. Experimental evidence suggests that activated suppressor cells elaborate at least two different soluble factors which independently modulate humoral and cellular immune reactions.

The immunoregulatory properties of a murine monoclonal antibody, anti-Tac are being studied. Experimental evidence suggests that anti-Tac reacts with the human T cell surface receptor for interleukin-2. In contrast, it does not interfere with B cell proliferation induced with EBV, a helper T cell independent polyclonal stimulant. Direct binding studies to define the relationship of anti-Tac and the human IL-2 were also performed, and the putative human IL-2 receptor has been purified and characterized. Recent studies indicate that this receptor is phosphorylated, suggesting that it is a transmembrane protein.

Understanding the mechanism of action of the antigen-specific immune response (Ir) genes, and the structures and specificity of the T and B cell receptors involved, are necessary to understanding the mechanism of genetic control of the immune response. Previous studies demonstrated that the serum antibody response and the cell proliferation response to sperm whale myoglobin were both controlled by the same two Ir genes mapping in different subregions of the H-2 complex. Each of these genes controls the response to different parts of the antigen molecule. When T cells from F<sub>1</sub> hybrid mice were tested with antigen and macrophage from high, low, or recombinant strain mice, the part of the antigen to which they responded depended on the source of the macrophage. Utilizing a newly developed in vitro culture system, the secondary antibody response was shown to be T-cell and macrophage dependent, and to be controlled by the same two determinant-specific Ir genes. This system has been used to study the cellular interactions in the mechanism of the Ir gene action. Experiments have demonstrated

that the Ir gene control is manifested in a restriction on T cell-B cell interaction, which correlates with the predominant B cell subset in this response. In other studies not involving Ir gene control, this subset required genetically restricted T cell help. It was found that the response to equine myoglobin was controlled by genes which are different from those controlling the response to sperm whale myoglobin. To examine B cell receptors for myoglobin, antibodies were prepared for high affinity monoclonal anti-myoglobin antibodies. The antigenic determinants on myoglobin recognized by three of these have been determined.

Several ongoing projects focus on better understanding the cellular mechanisms regulating normal mucosal immune responses and disorders of immune regulation in gastrointestinal disease. The B cells, which develop in Peyer's patches and migrate to secretory surfaces bear surface IgA. Cloned T cell lines have been developed from relevant lymphoid tissues, and studies are underway to assess the ability of these lines to regulate LPS-driven IgA immunoglobulin synthesis and secretion.

Peyer's patches (PP) cloned T cells appear to induce class-specific switching sIgM- to sIgA-bearing B cells, whereas cloned T cells lack this property. The PP switch T cells seem to operate as true switch cells governing the pathway of DNA recombination events. These data favor the concept that B cell development resulting in IgA expression involves preferential and class-specific differentiation from IgM-IgA, which is controlled by a new class of regulatory T cells, IgA-specific switch T cells. Peyer's patches are a source of IgA B cells because they are a repository of such switch T cells.

Prior studies demonstrated that primary biliary cirrhosis (PBC) is associated with a defect in the capacity to generate an autologous mixed lymphocyte reaction (MLR) and with decreased natural killer cell activity. Recent work suggests that PBC is due, in part, to a defect in an antigen non-specific regulatory loop which results in decreased suppressor T cell activity. The NK defect may be due to defective inducer cell function which is parallel to the suppressor T cell defect.

Other studies have been directed towards examining the mechanisms of action of cytotoxic mononuclear cells and their contribution to the host defense. Studies were continued to define the cellular requirements for the in vitro production of self-major histocompatibility complex (MHC) restricted cytotoxic T cells (CTL) immune to influenza viruses. Previous studies demonstrated that the actual killer cells and their precursors were contained within the total T-cell population. Recent studies have shown that the CTL precursors and the CTL killers themselves possess the same phenotype; however, the maturation of such precursors into effectors requires "help" or "amplification" by an additional subset of T cells..

Patients with a variety of immunodeficiency diseases have been studied for the capacity to generate such CTL in vitro. Patients with common variable hypogammaglobulinemia (hypo) and patients with x-linked hypo and isolated growth hormone deficiency produced influenza virus specific "self"-MHC restricted CTL normally, whereas patients with A-T and Wiskott-Aldrich syndrome (WAS) failed to produce immune "self"-MHC restricted CTL in vitro. In all "non-responding" patients this inability to detect CTL was due to the lack of CTL generation. The lack of immune "self"-MHC restricted CTL production in A-T and WAS patients may be related to their recurrent infections and high incidence of neoplasia.



Clinical research interest continues in assays of spontaneous monocyte mediated cytotoxicity. A reliable in vitro assay has been developed which measures inhibition of monocyte cytotoxic function by very small numbers of tumor cells. This assay correlates well with previous in vivo observations using peripheral blood from patients with malignant disease treated with x-irradiation. These studies imply that actual activation of cytotoxic monocytes may be induced by agents which are normally thought of as toxic (x-irradiation). Such activation may play an important in vivo role. The molecular requirements for the expression of synergistic cytotoxicity have been characterized using a model which measures the ability of human serum factors to activate human monocytes and lymphocytes, and to kill erythrocyte targets. Homogenous purified complement components have shown that C5 and Factor B are necessary and sufficient to induce lysis in the presence of activated human monocytes. Lymphocytes also exhibit killing and require additional C components. This research is important because it unites antigen dependent fluid phase lysis (alternate complement pathway) with antigen independent cell mediated lysis (NK and spontaneous cytotoxicity).

The study of the mechanism of action of growth hormone and insulin-like growth factors has been a continuing research focus. Previous studies have shown that MSA, an insulin-like growth factor, is a fetal growth factor produced by rat embryo fibroblasts. Recently it has been demonstrated that placental lactogen, a hormone homologous to growth hormone, stimulates the biosynthesis of MSA by the rat embryo fibroblasts. Additional studies demonstrated that the binding characteristics of the purified receptor for insulin-like growth factors are the same as the binding characteristics for whole cells. Studies of the metabolism of proline and porphyrin have continued. A metabolic cascade initiated by pyrroline-5-carboxylate (PC) has been defined that results in increased nucleotide formation. Researchers have shown that PC markedly stimulates the conversion of 6-mercaptapurine to 6-mercaptapurine monophosphate which is considered to be the active antitumor moiety. The use of PC as a possible adjunct to chemotherapy will be an area of emphasis.

The physiologic and/or pathophysiologic role of the proline cycle is being investigated further. "Stress states" e.g. higher temperature, anoxia, increased physical work, will be examined for proline cycling.

The kinetics of heme and hematoporphyrin uptake by malignant L1210 cells in culture and the porphyrin mediated killing of tumor cells by light have received further attention. Quantitative comparisons of the efficiency for tumor cell killing by photons of various wavelengths in porphyrin treated cells showed that 503 nm was the most efficient wavelength for tumor killing, both in terms of photon exposure of the cells, and in terms of photon absorption by tumor cells.

Hematoporphyrin uptake by tumor cells can be increased by exposure of the cells to succinylacetone, dibucaine and chloroquine. The amount of light required to kill L1210 cells in the presence of hematoporphyrin was decreased by addition of dibucaine or chloroquine to the medium, but was unaffected by antioxidants vitamins A and C, suggesting that photochemical destruction of tumor cells involves mechanisms other than generation of singlet oxygen. Tumor cells grown in the presence of succinylacetone were killed by smaller doses of light after addition of hematoporphyrin than those not exposed to succinylacetone.

## Dermatology Branch

The Dermatology Branch, (Dr. S. Katz, Chief) conducts both clinical and basic research studying the etiology, diagnosis and treatment of inflammatory and malignant diseases involving the skin and the host's response to these diseases. Recent studies have demonstrated that there are two types of immunologically relevant cells within the epidermis, the langerhans cells and keratinocytes. Each of these cells was shown to activate T cells in vitro. Work continues on the identification and characterization of antigens and antibodies involved in autoimmune blistering skin diseases, showing, among other things, that a pemphigus antigen is a glycoprotein of m.w. 130 kd. Studies are underway to characterize skin specific antigens which are identified by monoclonal antibodies. One of these is a unique, squamous epithelia specific, lamina densa antigen which is identified by KF-1 antibody.

Circulating antigen-antibody complexes have been implicated in the pathogenesis of a variety of diseases. The immune complexes which exist in Sjogren's syndrome, mixed cryoglobulinemia, mixed connective tissue disease, acute and chronic schistosomiasis, hepatitis and various types of cutaneous and systemic vasculitis, have been identified and partially characterized. Circulating IgA containing immune complexes have been demonstrated in patients with dermatitis herpetiformis, gluten-sensitive enteropathy and IgA nephropathy. Studies addressing the role of dietary protein in the induction of immune complexes are currently underway. Clinical investigations continue to evaluate safety and effectiveness of oral and topical synthetic retinoids in the treatment of skin cancer, disorders of keratinization and cystic acne. New and potentially more potent and less toxic synthetic retinoids are continually being sought.

Normal human cells have repair processes which rapidly and effectively repair DNA damage. Most patients with xeroderma pigmentosum (XP) have a marked impairment in the rate and/or efficiency of repair of DNA damage induced by UV-radiation or chemical carcinogens. Patients with XP are particularly susceptible to the carcinogenic action of UV-radiation. Understanding the relationship between DNA repair deficiency and skin tumor development would elucidate the role of DNA repair in carcinogenesis.

Work continues on the biochemical characterization of mammalian melanosomes. These studies are aimed at elucidating the mechanism of formation of these atypical proteins, as well as their importance to the immunology of melanoma and as possible immunotherapy agents. Many of the proteins in melanoma melanosomes are unique and are not found in normal melanin granules. In the murine system, a comparison of analogous proteins from normal and melanoma melanin granules, resolved a slight, but significant, difference of isoelectric points a difference in molecular weight and significant differences regarding four amino acids. Peptide mapping has revealed that amino acid sequences are deleted in 3 or more regions in the abnormal protein. It has been found that tumor-specific proteins similar to these can be found in the serologic fluids of melanoma patients and mice, and that large quantities of these proteins are shed from melanoma cells in vitro.

Papilloma viruses are a common cause of epidermal tumors in man. The determination of the transforming sequences of bovine papilloma virus (BPV) DNA represents a first step towards understanding how these tumors are formed. Results indicate that the transforming protein is encoded in a short region of the viral

genome and that transformation can occur independently of viral replication. The finding that interferon treatment can cure some tissue culture cells of BPV sequences and revert them to the normal phenotype provides an experimental basis for attempts to treat papillomavirus induced disease with this drug.

Studies of viral and cellular oncogenes have also made considerable progress. The molecular organization of the Harvey and Kirsten murine sarcoma viruses has been defined. Although Harvey and Kirsten viruses both encode crossreacting 21kd transforming proteins, their coding genes are derived from different cellular genes, and are part of a multigene gene family. Both of these genes are conserved in evolution, and are readily detected in human DNA. Four different p21 genes have been molecularly cloned from normal human DNA. The structure of one human Harvey type gene is very similar to that of a rat Harvey type gene. This normal human gene also has the capacity to induce oncogenic transformation of mouse cells when the p21 protein encoded by this gene is expressed at high levels.

The study of chemistry, structure and biosynthesis of mammalian epidermal keratin filaments continues. Comparisons of the polyacrylamide-gel electrophoretic profiles of the keratin subunits from abnormal and normal human epidermis show prominent differences in numbers and mobilities of bands, suggesting differences in the chemical structures of these proteins. Filaments assembled *in vitro* from psoriatic epidermis are also abnormal, and interestingly, form 'paracrystalline' structures consisting of several filaments associated side-by-side in an apparently ordered manner.

Normal mouse epidermal cells grown in monolayer culture can be made to synthesize the normal complement of keratin polypeptides. These proteins have been isolated and characterized and are currently being used as markers in studies of *in vitro* carcinogenesis. Preliminary experiments have revealed marked alterations in the synthesis of the keratin proteins during treatment with carcinogens such as TPA and other growth effectors such as vitamin A.

#### Immunology Branch

The Immunology Branch (under recently appointed Acting Chief, Dr. D. Sachs) carries out laboratory investigations in the following areas: 1) regulation and control of immune response; 2) structure and function of cell surface molecules; 3) transplantation biology; and 4) tumor immunology including clinical studies in immunotherapy. In addition, the Immunology Branch maintains a fluorescence activated cell sorter facility which is involved integrally in many internal research projects and in a number of collaborative investigations.

The investigation of the generation and regulation of T cell dependent responses to both conventional and alloantigens, and the study of the mechanisms of interaction among T cells, B cells and accessory cell populations have been of primary research interest. Monoclonal T cell populations specific for the antigens (T,G)-A--L and KLH have been examined. Such T cells were found to recognize both antigen and self H-2. Additional studies have shown that developmentally distinct B cell subpopulations are activated by helper T cells in genetically distinct ways.

Further investigations have been directed toward understanding the role of the major histocompatibility complex in regulation and restriction of T cell



mediated and effected immune responses against chemical haptens and infectious viruses. Studies of genetic control of human CTL responses against influenza-virus infected autologous cells have also been performed. These studies may elucidate the role of HLA antigens in regulating human T cell immunity against infectious viruses, and may help to define the cellular components involved in this response.

Studies of the structure and function of cell surface molecules continue. The molecular features of membrane damage induced by immune mechanisms have been studied in an attempt to understand how lymphocytes kill foreign cells. Mechanisms involved in the triggering and regulation of immunocompetent cells and the role that cell surface molecules play in this triggering are being investigated. Recent findings indicate that the  $Fc\gamma$  receptors of B lymphocytes interact with: a) the lymphocyte cytoskeleton, b) Ia antigens and Lym antigens, c) surface, IgM, and d) surface IgD. Each of these interactions is distinct, specific, and non-random. In addition, monoclonal antibodies specific for  $Fc\gamma R$  induce B lymphocytes to both proliferate and secrete antibody. This response is specific and does not require T lymphocytes. This is the only antibody specific for a B lymphocyte receptor which triggers function, thus suggesting that B lymphocyte  $Fc\gamma$  receptors are central to B lymphocyte activation and immunoregulation. Additional work is underway to characterize the mechanisms of immunoregulation of antibody synthesis and secretion, particularly Ir genes and idiotype networks. Recently, a system has been developed in which, soluble antibody responses to the synthetic polypeptide (T,G)-A--L can be generated and detected in vitro. Responses are antigen dependent and specific, regulated by H-2 linked Ir gene. The anti-idiotype reagent stimulates function of both primed T helper lymphocytes and of primed B lymphocytes (antibody secretion which occurs in the absence of T lymphocytes).

Studies in the Transplantation Biology Section have been directed toward understanding the structure and function of products of the major histocompatibility complex (MHC) and manipulations of the immune response to these products. A large number of hybridoma cell lines producing antibodies to H-2 and Ia antigens have been produced and characterized. These antibodies have been used to further subdivide products of the MHC. In addition, anti-idiotypic antibodies against these hybridomas have been produced and the effects of such anti-idiotypic reagents on in vitro and in vivo parameters of histocompatibility have been examined.

Three herds of miniature swine, each homozygous for a different set of histocompatibility antigens at the MHC have been developed. Purification and characterization of the major histocompatibility antigens of this species, and isolation and characterization of peptides from these antigens for sequence analyses and for assessment of immunologic reactivity are underway. Two new recombinants within the MHC have been detected within the miniature swine herd. Both recombinants involve separation of the mixed lymphocyte culture (MLC) stimulatory locus from the serologic loci. Transplantation studies aimed at determining the relative importance of individual MHC loci are now in progress using these new recombinant lines. The alloreactive T cell repertoire has been analyzed for responses to two categories of alloantigens. The findings suggest that the T cell repertoire for alloantigens, like that for conventional antigens, may be both MHC restricted and environmentally modified. Studies are continuing on the role of HLA gene products in recognition of foreign antigen by human T cells. The data indicate considerable diversity among serologically indistin-

guishable molecules, and suggest that individual HLA-A3 molecules may express more than one site for recognition by MHC restricted T cells. The major emphasis has been on further characterization of a new HLA gene, designated "SB" or "secondary B cell" gene. Studies have been carried out to assess the relevance of these new SB markers in multiple sclerosis and dermatitis herpetiformis. Such studies may extend our understanding of the biological importance of these MHC linked antigens.

Recombinant DNA technology has been used to study the genome organization of the major histocompatibility complex. Ten to 15 MHC genes have been demonstrated in the pig genome and many of these have been isolated. A series of genomic clones containing MHC-homologous DNA sequences has been isolated. One of these clones has been studied in detail, both with respect to its DNA sequence organization and regulation of expression. In addition, a detailed analysis of MHC-linked but non-coding sequences has revealed that MHC genes are embedded in clusters of repetitive DNA which recur non-randomly in all of the MHC gene-containing clones isolated to date. These flanking sequences may be involved in the regulation of MHC evolution, generation of polymorphism or expression.

Studies in tumor immunology have been directed toward identification of factors which influence host cytotoxic cell responses against syngeneic tumors. Effector cells mediating broadly reactive anti-tumor cytotoxic activity have been induced under syngeneic conditions *in vitro*. These are closely related to natural killer cells, as judged by expression of cell surface differentiation antigens and target cell specificity, and the response is controlled by multiple genes.

#### Macromolecular Biology Section

The Macromolecular Biology Section in the Immunology Program has focused its research efforts on studies of specific macromolecular changes on the surface of mammalian cells, relationship of such changes to certain normal (differentiated) cell surface functions, and their role in the appearance of cellular tumorigenicity. Investigators have found that a 55,000 MW cellular protein which is "induced" after SV40 infection of cultured mammalian cells, is also present in embryo primary cells which were not exposed to the SV40 virus. The mouse embryo protein and the "induced" protein in SV40 transformed mouse cells when labelled with <sup>35</sup>S-methionine have virtually identical 2D tryptic peptide fingerprints. Mouse, rat and hamster embryo <sup>35</sup>S-labelled 55K proteins and the 55K SV40 induced proteins from the same species are structurally identical, but have species divergence approximating the expected evolutionary order and divergence. It is likely that the 55K protein has an important evolutionary conserved, cellular function. Further studies have shown that the 55K protein is being expressed constitutively in normal embryogenesis.

#### Laboratory of Immunobiology

Research in the Laboratory of Immunobiology, (directed by Dr. T. Borsos) has focused on studying the mechanisms of the effector arm of the immune system. In the Humoral Immunity Section, experiments have been directed towards investigating the mechanisms by which immunoglobulins acquire complement fixing and activating properties, the relation of chain structure to binding and activity of complement components, and the mechanism of the damage producing steps. The biochemical events that govern the maintenance of cell integrity and cell sus-



ceptibility in face of humoral immune attack are also being investigated. Monoclonal antibodies have been produced to cell antigens and to drugs to study the mode of action of antibodies at cell surfaces and the utilization and metabolism of drugs.

A major effort in the past year has been directed at elucidating the conditions that confer binding and activating properties upon immunoglobulin. Past studies showed that IgM anti-methotrexate (MTX) antibodies bound to cells in three different forms, and that the ratio of the different forms depended on the density of MTX coupled to the cell surface. In contrast to IgM, individual IgG molecules are incapable of activating C sequence even when combined with appropriate antigens. Although soluble IgG can interact with the antibody binding subcomponent of C1, such interactions cannot be shown to occur at cell surfaces. Work in this laboratory has shown that acquisition of C1 binding by IgG depends on the proximity of at least 2 IgG molecules not more than 30 nm apart, and that C activation most probably depends on the angle of the Fab arms of the IgG molecules. Both of these functions depend on the distribution and density of hapten.

In concurrent studies on the mechanism of cell lysis by IgM antibodies and C, the hemolytic efficiency of rabbit anti-Forssman IgM antibody was shown to depend on the source of C components. With whole guinea pig C one in 10 IgM molecules was hemolytic; with purified guinea pig components C1, C4 and C2 and with guinea pig C3-C9, one in three was lytic. Evidence was obtained that the block occurs at the C4 level, indicating that C4 was a pivotal molecule in the classical pathway of C activation. The form in which C4 appears in the sequence depends upon the Ig-C1 complex, which, in turn depends upon the distribution and density of the hapten interacting with the Ig. Further studies suggest that during the activation of the classical C sequence, C4 has to bind to cell receptors at some distance from the IgM molecule, and that C4 has the capability of interacting with and binding C2 to generate the C42 enzyme. Monoclonal antibodies to methotrexate were prepared and characterized. The efficiency of producing hybridomas with FO myeloma cells was shown to depend strongly on the molar ratio of drug to the carrier protein. By manipulating the epitope density it was possible to influence the number of fusion products secreting monoclonal antibodies with desired specificity. Similarly, mice immunized with the high drug/carrier immunogen had significantly higher levels of antimethotrexate in the plasma although the binding affinity of the antibodies was the same regardless of whether the mice had been immunized with an immunogen containing a high or low level of methotrexate.

Three IgG<sub>1</sub>, two IgG<sub>2a</sub>, and two IgG<sub>2b</sub> monoclonal antibodies have been produced in the ascites fluid of mice at concentrations in the range of 2-3 mg/ml. The IgG<sub>1</sub> antibodies bind neither complement C1 nor protein A of S. aureus, whereas all the IgG<sub>2a</sub> and IgG<sub>2b</sub> antibodies bind both proteins. Antibodies produced in the ascites may prove to be useful standard reagents in immunological determination of methotrexate levels in cancer patients undergoing chemotherapy with methotrexate.

It was previously shown that the number of human blood monocytes responding to chemotactic stimuli under optimal conditions in vitro was less in cancer patients than in normal controls or hospitalized patients without cancer. Questions about the nature of this defect have led to studies of monocyte subpopulations, chemotaxin receptors, maturation dynamics and factors affecting chemotactic response.

Among normal human blood monocytes, 20-40% respond to chemoattractants. In the case of the peptide attractant, (FMLP), the non-migrating monocytes lack the receptor for the attractant. An attempt is now underway to determine whether absence of FMLP binding by non-migrating monocytes is an isolated difference or whether these cells differ in many respects from the morphologically similar migrators. Populations of migrating and non-migrating monocytes were obtained by use of the chemotaxis separation chamber. Phagocytic capacity for IgG coated sheep erythrocytes was the same for the two populations. Migrating monocytes exhibited a respiratory burst to FMLP, whereas the non-migrators had little or no response. Similarly, the non-migrators had a much lower response to phorbol myristate acetate (PMA). Thus, in addition to lack of the FMLP receptor, these subpopulations differ biochemically in other respects.

C5a, derived by activation of the fifth component of complement, is the major chemoattractant produced from serum. Modification of C5 in a process that does not involve complement activation results in potent chemoattractant activity which is heat-stable. The precursor, or one of the reactants involved in attractant formation, is heat-labile. Neither classical nor alternative complement pathways appear involved, suggesting that at local inflammatory sites enzymatic modification of C5 may generate chemotactic activity without complement activation.

Characterization of activation signals and the mononuclear phagocyte subpopulations that respond to these signals to activate the cytotoxic response of macrophages constitute other major areas of investigation in this laboratory. Macrophage activation by lymphokines (LK) for nonspecific tumor cytotoxicity requires multiple reactions. Stable, noncytotoxic cellular intermediates between immature mononuclear phagocytes and fully activated tumoricidal macrophages can be identified. Optimal cytotoxicity occurs with cells treated for 4 hours with low concentration of LK and then triggered 1 hour with high concentrations of LK.

Supernatant culture fluids from a phorbol myristate acetate (PMA)-stimulated subline of the murine EL4 thymoma activated inflammatory macrophages for non-specific tumoricidal activity in vitro. The titer of activity in cytokines from PMA-stimulated EL-4 was similar to that found in LK and the time courses were identical. Both EL-4 and LK activities were nondialyzable, destroyed after 10 minutes at 100°C and they could be separated from IL-2 macrophage-granulocyte colony stimulating factor or from the B cell growth factors. Further physicochemical characterization of the EL-4 cytokines showed 2 distinct activities that activated macrophages for cytotoxicity. These results suggest that the EL-4 cell line may produce one or more of the macrophage activation factors present in LK. Nonspecific effector function by LK-activated macrophages appears to be controlled by both the physicochemical nature of the LK mediator and by the time period for which effector and target cells are exposed to LK.

The in vitro interaction of the effector arms of the immune system with cell surface factors has in vivo counterparts. Studies have continued on development of methods for modifying host response to weakly or nonimmunogenic tumor cells.

To develop an approach to control of non-immunogenic tumors, fibrosarcomas were infected in vitro with murine leukemia viruses and the consequences of this infection on tumor growth, immunogenicity and cell viability were measured. Murine leukemia virus infection had no detectable cytopathic effects on the tumor

cells. Animals injected with virus-infected tumor cells developed tumors which regressed. In contrast, uninfected tumor cells grew progressively, leading to the death of the recipient. Further studies indicated that tumor eradication occurred without development of immunity to intrinsic tumor antigens. Studies of the basis of adoptive transfer of tumor immunity continue to elucidate the types of cells that are important in the donor population and to identify the cells that the host contributes.

### Laboratory of Cell Biology

A continuing focus of research activity in the Laboratory of Cell Biology, (Dr. L. Law, Chief) has been the study of mouse plasmacytomas, the immunoglobulins they synthesize, and the genes that control this synthesis. Karyotypic analysis has shown that these plasmacytomas frequently have a translocation of a fragment of chromosome 15 onto one of the chromosomes that contains the structural genes for light or heavy chains of immunoglobulin. This suggests that chromosome 15 contains an oncogene specifically associated with lymphocyte malignancies which may be activated when it is translocated next to an active promoter for the Ig genes.

Three-dimensional structure of the antigen-binding region of myeloma proteins is being studied. It has been shown that anti-phosphocholine and anti-galactan antibodies have very differently shaped binding sites which determine their immunological specificities. Studies of phosphocholine binding Ig heavy chains in BALB/c and C3H mice have demonstrated that there are two allelic  $V_H$  genes that can be used in this response. The  $V_H$  sequence of a hybridoma protein that binds phosphocholine differs from these two allelic genes in a way that suggests that "gene conversion" may play a role in the generation of antibody diversity. The distribution of these allelic forms of phosphocholine binding  $V_H$  and  $V_L$  genes as well as the families of  $V_H$  and  $V_L$  genes for antigalactan antibodies are being determined in many strains of inbred and wild mice using cDNA probes. The differences in stability of these two gene families during interdependent evolution in wild mouse populations will provide important insights into the genetic mechanisms that conserve the function of these biologically important molecules.

Wild mice have been screened by Southern blotting for the presence of the genes of mammary tumor virus. Many strains of wild mice were found to be completely free of these genes, while some have only a single provirus in the genome and others just the long terminal repeated sequence of this virus. Further studies are underway to determine the incidence of mammary tumors, and to test the promoter-insertion theory of mammary tumorigenesis.

The organization and expression of the constant region genes of the Ig heavy chains has focused on the mouse IgD  $\delta$  heavy chains. Investigators in this laboratory have cloned  $\delta$  cDNA and genomic  $\delta$  and determined the structure of the introns, exons and 3' gene segments encoding secreted and membrane forms of  $\delta$  chain. In plasmacytomas and normal spleen RNA an unexpected multiplicity of membrane  $\delta$  mRNAs was found. IgD appears to be an important multifunctional B cell receptor, and the multiple  $\delta$  mRNAs may indicate that there are multiple forms of  $\delta_m$  protein, each capable of a different receptor function.

A first successful isolation of amphotropic type C retroviruses from sources other than wild mice was accomplished. One of these two new isolates proved to be a recombinant virus which derived its gag gene from AKR-like MuLV and env gene



from the endogenous amphotropic virus. This virus emerged from a Rauscher-MuLV induced tumor, RBL-5, raising interesting questions concerning its role in the viral activation and recombination processes.

The regulation of expression of H-2 and beta-2-microglobulin in trophoblast cell lines from murine placentas was examined. It was found that the very low expression and synthesis of H-2 and beta-2-microglobulin in these cells is due to the transcriptional control as is the case with embryonal carcinoma (EC) cells. Natural Killer (NK) cells, but not alloimmune T-cells, were able to kill trophoblast cells.

A high frequency of co-transfection of transformation and TSTA, at least among the primary transfectants, has been observed. Further results suggest that in the Meth A sarcoma a transforming gene and a genetic determinant of TSTA are intimately related. Meth A DNA cleaved with restriction enzymes retains transforming capacity. This DNA fragment appears to be of a size appropriate for molecular cloning. The frequency of co-transfer is much lower and not all immunogenic clones have been assayed for specificity. It will be of interest to determine if this isolated determinant is related to viral onc genes and the relationship of this determinant to our 75K TSTA. Attempts are being made to isolate and purify the TSTA of RBL-5 and RMu-LV induced T cell leukemia. Higher yields of TSTA are now available and purification is proceeding. Two major bands (~75K and 65K) on SDS-PAGE have been visualized. These materials along with their dimers are very immunogenic against other FMR-induced lymphomas but not against G-MuLV-induced or radiation induced leukemia. The 75K material is not related immunologically to the 75K Meth A isolate.

Evidence suggests that the regulation of major histocompatibility antigen expression is due to transcriptional control of both H-2 and  $\beta_2$  microglobulin. Two cloned cell lines derived from the whole placenta of two strains of mice were examined. Very low levels of both H-2 and  $\beta_2$  microglobulin were detected on the cell surface, correlating with the low levels of mRNA observed for these proteins. The cloned cells have been shown to be trophoblast. The low level of histocompatibility antigens in this particular region may be an important factor in the survival of the fetus as an intrauterine semi-allograft.

Much research effort has been directed towards studying the mechanism by which T-lymphocytes recognize antigen only in association with macrophages of a particular H-2 type. The specificity of T lymphocyte antigen recognition has been examined by using a family of peptide antigens. Present studies have shown that there is specific interaction between the macrophage and antigen. Currently investigations are underway to define the sites of T cell-antigen and MHC-antigen interactions.

Methylcholanthrene-induced sarcomas are known to carry a distinctive antigen (TATA) which can impart transplantation immunity to the tumor of origin by prior immunization with that tumor or with a soluble form of the antigen derived from it. A TATA with an apparent molecular weight of 75,000 has been isolated from the cytosol of the BALB/c sarcoma Meth A, and has been purified. A second protein having a molecular weight of 75,000 was isolated from the cytosol of the methylcholanthrene induced sarcoma, CI-4. The antigens purified from Meth A and CI-4 sarcomas appear to be closely related but non-identical proteins. Studies are underway to investigate the primary structures of these antigens to determine the relationship between individual TATAs from different sarcomas. Additional

studies are now in progress to determine the molecular organization of the genes involved in the expression of these antigens.

Investigators have continued to examine the mechanism by which cells generate slow rhythmic pacemaker potential (10-40mV fluctuation with period 5-10 min). Input resistance does not change during the pacemaker cycle, therefore, it is not due to change in membrane ion permeability. Experimental data suggest that intracellular  $\text{Na}^+$  is necessary for initiation of the pacemaker because: 1) injection of  $\text{Na}^+$  indicates pacemaker potentials; 2) amiloride does not block pacemaker potentials initiated by injection of  $\text{Na}^+$ , and 3) pacemaker potentials are initiated by cessation of amiloride which allows reestablishment of  $\text{Na}^+/\text{H}^+$  exchange.

Studies continue on the effects of interferon on murine retrovirus. When chronically infected SC-1 cells were treated with highly purified interferon for 24 to 48 hours, there was approximately a 100-fold reduction of infectious virus after the interferon treatment, but only a ten-fold reduction in the number of particles, indicating a significant production of noninfectious virus. After a 24 hour exposure to interferon, the virions contained an 85 K dalton glycoprotein of non-viral origin which was present in excess of the amount of the viral envelope glycoprotein, gp70. Virus particles produced from cells treated with interferon from 32-48 hours were nearly devoid of gp70 and contained measurably lower quantities of p30, confirming the observation that gp70 is essential for infectivity. It is probable that gp70 is not assembled into particles and is released from p15E/p12E following proteolytic cleavage of pPr 85env. The molecular basis for this failure of incorporation of gp70 into the MCF class of retroviruses is not as yet known, but it seems quite possible that restriction in virus assembly is not exclusively localized to virus maturation sites, but may occur at other locations in the plasma membrane of the host cell, while leading to the same end result, the failure of incorporation of gp70 into virions.

Recent studies on the genetic mechanisms of carcinogenesis demonstrated that murine type-c leukemia virus of low pathogenicity, when recombined with a subset of host cellular nucleotide sequences gave rise to competent oncogenic viruses. Four recombinant DNA clones of rat endogenous leukemia helper virus (RaLV) genomic DNAs have been established. Restriction maps were deduced for two molecularly cloned RaLV DBAs of laboratory and feral origins. Functional organizations of both these RaLV DNAs were established. Further analysis has identified some nucleotide sequences of these RaLVs that are highly divergent and others that have been conserved in evolution.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01-CB-08900-17 OD Formerly Z01-CB-05006-16
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Lymphocyte Cell Surface Antigens, Normal and Neoplastic

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

P.I.:	D. L. Mann	Senior Investigator	OD	NCI
Other:	W. Blattner	Section Head, Family Studies	EEB	NCI
	M. Green	Senior Investigator	EEB	NCI
	A. Muchmore	Senior Investigator	MET	NCI
	S. Broder	Acting Deputy, DCT	MET	NCI
	C. Harris	Lab Chief	LHC	NCI
	D. Volkman	Senior Investigator	LIR	NIAID
	G. Chrousos	Senior Investigator	CH	NIADDDK

COOPERATING UNITS (if any)

LAB/BRANCH

Office of the Director, DCBD

SECTION

INSTITUTE AND LOCATION

NCI, NIH Bethesda, MD 20205

TOTAL MANYEARS:

1.0

PROFESSIONAL:

1.0

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☒ (b) HUMAN TISSUES

☐ (c) NEITHER

☐ (a1) MINORS

☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Biologic, functional and chemical characterization of cell surface structures of normal lymphoid and malignant cells are under investigation. Antigen (HLA) controlled by the major histocompatibility complex (MHC) have been assessed in diseased populations and at risk families. Results indicate that several inter-acting genes and gene orientation predispose to disease states. Immune response, specifically directed to the insulin molecules appears to be controlled by the human MHC. Immunoprecipitation analysis of antigens controlled by the MHC DR gene region suggests at least 3 gene products which are complexes of at least 3 different heavy and 6 light chains. Intact antibodies reacting with these structures suppress in vitro lymphocyte stimulation by antigen and Ig productions. Cultured lymphoma cell possessing the HTLV virus express inappropriate HLA antigens. Naive fetal cells are induced to express these HLA antigens when infected with HTLV. Lymphocytes from homosexual men have altered helper/suppressor ratios with decreased total helper cells. This alteration appears associated with amyl nitrate use.

## Project Description

Objectives: The objectives of these studies are as follows: a) To identify genes in the major histocompatibility complex which have an association with disease states; b) to identify potential immune response genes in man; c) to assess the biologic function of different cell surface structures in relationship to immune function of lymphocytes; d) to isolate and purify gene products of the major histocompatibility complex for structural analysis; and e) to identify lymphocyte cell surface markers using monoclonal antibodies which react with functional subsets of lymphocytes. These objectives are applied to studies in malignant and premalignant states.

Methods Employed: Gene products of the major histocompatibility complex are identified by dye exclusion cytotoxicity tests. The frequency of HLA allo-antigens in a disease population are compared with frequencies in a normal population and/or related disease population by Chi square analysis. In family studies haplotype association with the disease was determined by lode score analysis.

The generation of suppressor cell function using anti-HLA-DR sera and monocytes was performed using isolated monocytes exposed to antigen (SKSD, Candida tetanus toxoid) washed, reacted with antisera and incubated with autologous or allogeneic T lymphocytes. Tritiated thymidine incorporation was measured after 5 days. Suppression of Ig synthesis was measured by inhibiting anti-B cell serum with peripheral blood lymphocytes and the cultures stimulated with the mitogen pokeweed. Levels of IgG, IgA and IgM in supernatants from these cultures were measured by a radioimmunoassay.

Cell surface antigens have also been investigated employing flow micro-fluorometry using the fluorescence activated cell sorter and monoclonal antisera.

Studies of the molecular characteristics of human B cell alloantigens were performed by immunoprecipitation of labelled cell membrane components and with chromosomal gel electrophoresis.

Major Findings: HLA and Disease Association: Studies in the Pima Indians resulted in the following observations. HLA alloantigen and gene frequencies were compared in the Pima and Papago Indian population and mixtures. Some distinct HLA antigens were found in each population. The results indicate a genetic distinction in these 2 tribes that are geographically and culturally related. HLA-A2 allele frequency was observed to be increased in the insulin independent diabetic population in Pimas. HLA-B27 positive Pima Indian men are at greater risk for the development of sacroileitis indicating that this association is not limited to the Caucasian race.

Studies of HLA types in Takayasu's arteritis patients demonstrated an increase in HLA-MB3 antigens in the disease population.

Families with several individuals with the attenuated form of CAH were studied for HLA antigen (haplotype) association. Family members could be biochemically "phenotyped" with ACTH challenge. The gene for this form of the deficiency was mapped in chromosome 6 in the MHC. Several unique families where both the attenuated form and the congenital form of the disease was found allowed in the construction of a 2 gene hypothesis (structural and regulatory) for this disease.

**Immune response regulation:** Antisera specific for HLA-DR determinants was used to study monocyte functions in *in vitro* measurements of the immune response. Whole IgG antibodies induced suppression of response while F(ab)<sub>2</sub> fragments of the antibody did not. The results demonstrate that blocking of antigen presentation is not the only mechanism for anti-Ia like antibody inhibition of immune reaction. Studies carried out in diabetic populations receiving insulin demonstrated a HLA associated genetic restriction in lymphocyte stimulation response to pork insulin, beef insulin proinsulin (beef and pork) and protamine. The results demonstrate that the human immune system can recognize and respond to the single amino acid difference in beef and pork insulin and that this recognition is controlled by the human MHC.

**Biochemical analysis of HLA gene products:** Immunoprecipitation studies of HLA-DR gene products with alloantisera detecting these antigens demonstrated molecular differences between HLA-DR and the MB antigens resolving the question of one or more genes controlling expression of the antigenic determinants. Comparison of the products of immunoprecipitation with antisera detecting different MB, MT and DR antigens suggest that the antigenic determinant of DR is on the light chain and MT, MB determinants on the heavy chain of the dimeric molecules. This has been an important advance in our understanding of the molecular and genetic relationships of the multiple antigens coded for by the HLA-D region of the MHC.

**T-cell markers in normal premalignant and malignant conditions:** Studies were carried out in normal male homosexuals in an area endemic for the Kaposi's sarcoma examining T lymphocyte markers. Alterations in helper/suppressor lymphocyte ratios were observed in the individuals frequently using amyl or butyl nitrates. (Usage of this drug is high in patients developing the sarcoma.) These results demonstrate potential altered immune functions which is drug related and may be a predisposing factor in the development of the malignancy.

Inappropriate expressions of HLA alloantigens (HLA-A, B, C but not DR) was found in cultured lymphoma cells that have detectable human T cell lymphoma virus (HTLV). B lymphoid cells from this patient had no detectable virus and no inappropriate expression of HLA. The HTLV lymphoid cells were examined for their antigenic cell surface profile. Lymphoma cells from six patients with this disease demonstrated the same cell surface antigen profile (not HLA) and inappropriate expression of an HLA-B5, 35 antigen. Expression of this antigen(s) was induced in normal cord blood T lymphocyte co-cultured with HTLV cell lines when the fetal T cells became virus positive. The results suggest that either this virus (provirus) carries the genetic information for this HLA antigen or that the virus integrates in the MHC of the human 6th chromosome and induces the expression of new gene products.

Significance to Biomedical Research and Program of the Institute: Identification of human alloantigens together with a description of the fine structure of the genetic complex is necessary for the understanding of the relationship of the MHC to disease states. Our studies of the various disease entities described above suggest that multiple genes in the MHC are associated with the disease entity or a manifestation of the disease. Studies in the diabetic population where specific allergic manifestations to insulin or insulin components have been identified, strongly indicate the presence of a specific immune response gene in man. These studies enhance the knowledge of human immunologic control which will lead to a better understanding of alterations of this control which might effect cancer susceptibility or control.

The above statements become relevant in our preliminary observations in the HTLV containing cells. It appears that this virus may be integrating in the MHC and thus may be responsible for abnormal cellular proliferation and potential alteration of immune response.

Proposed Course of Project: The studies are now and will continue to be directed primarily to families wherein more than one member has a malignant disease condition. The goal is to determine the potential for cooperative gene effects in the role of the pathogenesis of the disease. Families in which there are multiple cases of malignancies will be HLA typed and studied for in vitro immune response capability. In addition, lymphocytes from the family members will be studied for cell surface markers using monoclonal antibodies that are reported to differentiate functional subsets of lymphocytes. Information obtained from this multidirectional approach will be used to attempt to determine the role of the MHC and other genes in immune responsiveness and to attempt to define immune alteration in patients with malignancies. These studies will be performed in collaboration with the family studies section of the Epidemiology Branch, NCI.

Studies will continue in attempts to define specific biologic function of the molecular structures bearing the human DR antigens. These studies will be directed towards understanding the role of these structures in activating or suppressing immune response. These studies will be extended to patients with malignancies to amplify our observations that sera from acute leukemia patients contain substances (non-cytotoxic antibodies?) that induce suppression when reacted with leukemia cells.

Further characterization of the molecular structure of HLA-DR antigens will be undertaken. The specific goals will be to isolate and chemically characterize the molecules that bear the different antigenic determinants.

Studies will be continued to determine the relationship of the HTLV and the major histocompatibility complex in relationship to cell surface antigen expression and possible genetic control of infection.



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## SUMMARY

Annual Report of the Laboratory of Biochemistry, National Cancer Institute  
October 1, 1981 to September 30, 1982

### I. INTRODUCTION

Research has continued at a good pace during this past year. Much interesting work was accomplished and submitted for publication, and the staff of the Laboratory participated in a variety of meetings. Dr. Martin Rosenberg received the Fleming Award this year, in recognition of his leadership in research. Several important changes occurred in the Laboratory. Dr. Igor Dawid left the Laboratory to become Chief, Laboratory of Molecular Genetics, National Institute of Child Health and Human Development. Dr. Martin Rosenberg moved to Smith Kline Beckman (SKB) where he will build a research group to undertake fundamental studies in molecular biology. These two individuals have contributed to science in major ways here and also helped maintain the general level of excellence in the Laboratory. Both of them are leaders in the field of molecular genetics and biochemistry. Through their own enthusiastic participation as well as that of their younger colleagues, they benefited the work of many groups within the Laboratory, and their departure will be deeply felt.

The gloom engendered by these departures is ameliorated by the addition to the Laboratory of promising young investigators. Dr. Dean Hamer was converted to tenure this year and is proceeding with his active and fruitful research. Dr. Carl Wu and Dr. Cary Queen came from postdoctoral positions at Harvard and MIT, respectively, to initiate their independent scientific careers in this Laboratory.

The Wednesday seminar in our library has again offered an interesting roster of guest speakers and continues to attract scientists from all over the NIH. The visitors often spend considerable time in discussion with Laboratory members thus enriching our viewpoints and knowledge. The Laboratory "Show and Tell" is a weekly occasion at which members of the Laboratory take turns presenting their current research. It has not been as lively as we would like and next year the format will be reorganized to try to foster more discussion among individuals with very different areas of interest.

The limitations on staff fellowship positions along with the general cut-backs in available postdoctoral fellowships has had the effect of increasing the percent of foreign postdoctoral fellows in the Laboratory. The quality however remains high, and these young people contribute enormously to the activities and general atmosphere.

### II. BIOCHEMISTRY OF GENE EXPRESSION SECTION (Dr. E. B. Thompson, Chief)

Dr. Thompson and his colleagues are examining the biochemical and cellular actions of hormones with emphasis on steroids. Two general approaches are taken. This paragraph summarizes the first, which is the regulation of growth hormone and prolactin production in a rat pituitary adenoma cell line (GH<sub>3</sub>) and in hybrids between GH<sub>3</sub> and a mouse cell line (LB82) which does not express either gene. The hybrids are likewise non-expressing. cDNA probes for both

rat growth hormone (GH) and prolactin (Prl) were used to isolate the corresponding genes. Analysis by digestion with restriction endonucleases followed by Southern blots, showed that both rat and mouse GH and Prl genes are in the hybrid cells. The rat gene seems intact at this level of resolution. RNA analysis by translation assays and Northern blots shows that little or no GH RNA is produced in the hybrids; thus the dominant (mouse) L cell regulation probably acts by preventing rat GH gene transcription. Microinjection of many copies of rat GH gene can overcome this dominant negative control. Preliminary results suggest that the loss of prolactin synthesis in the hybrids is by a similar mechanism. Portions of the GH gene have been cloned into two vector systems and used to transform cells. The transformants will be examined for hormone-controlled GH expression. Also, appropriate synthesis of run-off transcripts from a GH gene fragment occurs in a cell-free system. These various systems will be used to pursue the study of the controls over expression of the GH and Prl genes. Two newly developed methods are of interest in this connection. The first is a simple modification of an older method for filter hybridization which now allows 1) the lipids and proteins to be extracted, by phenol for example; 2) the DNA to remain tightly bound; and 3) the filters to be reused after harsh extraction, without waiting for decay of  $^{32}\text{P}$  on previously bound probes. The second is a computer-assisted, image-intensification method for comparing large numbers of cDNA-containing colonies hybridized with probes from RNA populations of differing physiologic states. Both methods should be of general use.

The second main theme has been the study of glucocorticoid lysis of leukemic cells. Studies on an unusual phenylpyrazole glucocorticoid continue to look promising. This compound appears to lyse cells which are resistant to the standard glucocorticoids. Its toxicity in mice, its effect on several tumor cell lines, and binding of the  $^3\text{H}$ -labeled compound to cells are being studied. Also extensive characterization of a receptor-positive, lysis-resistant clone of the leukemic CEM cells, a clone of potential value for establishing the sequence of events involved in steroid-provoked cell lysis, has been done. Finally, the timing of cell death after steroid administration to several independent clones of CEM cells is being studied. The clonal variation seen is of possible significance for clinicians planning ALL therapy.

### III. BIOSYNTHESIS SECTION (Dr. E. L. Kuff, Chief)

The Section has three independent research groups.

A. (Dr. Beverly Peterkofsky and coworkers). This group has pursued studies on the regulation of collagen biosynthesis in normal and transformed cells. The role of ascorbic acid in collagen metabolism is being reinvestigated using a classical experimental system, the scorbutic guinea pig. Under conditions of acute ascorbic acid deficiency, collagen synthesis in the excised calvarial bone is decreased relative to total protein synthesis. Several observations suggest that this effect may be unrelated to the role of ascorbate as cofactor in proline hydroxylation. For example, decreased proline hydroxylation, but not decreased collagen synthesis, can be returned to normal by treatment of bone from scorbutic animals with ascorbate *in vitro*. Conversely, addition of  $\alpha,\alpha'$ -dipyridyl inhibited proline hydroxylation in bone from scorbutic animals without decreasing collagen synthesis. Pair-feeding experiments indicate that the decrease in collagen synthesis in bones from scorbutic animals is secondary to decreased food intake and a negative nutritional balance rather

than a direct effect of ascorbate deprivation on collagen metabolism. This group has also continued its studies on the modification of collagen phenotype by transformation. It had been earlier shown that the elevated type III to type I ratio characteristic of KIMSV-transformed fibroblasts is retained when cells transformed by a temperature-sensitive virus strain are shifted to the non-permissive temperature. Recent work has now shown that this effect cannot be explained by differing half-lives of the type III and type I procollagen mRNAs. Metabolic studies were also carried out on a series of virally and chemically transformed fibroblasts all derived from the same clone of 3T3 cells. The transformed lines differed widely in their rates of lactate production and glucose uptake; increases in these parameters are not an obligatory consequence of neoplastic transformation.

B. (Dr. Samuel Wilson and coworkers). This group has continued its investigation of the enzymes and accessory proteins involved in DNA synthesis. Structure-function relationships of *E. coli* DNA polymerase I large fragment were evaluated using a newly developed procedure for covalent modification of the enzyme protein with pyridoxal 5-phosphate (PLP). The data suggest that a single lysine residue in an essential dNTP-binding site is one target for PLP modification. Studies have been undertaken to localize this lysine residue in the primary sequence of the enzyme. With regard to the structure of the mammalian DNA polymerases, recent results of this group emphasize the presence and abundance of a 120,000-M<sub>r</sub>  $\alpha$ -polymerase catalytic polypeptide in mouse myeloma cells and several other mammalian cell types. The group has also observed that the in vitro translation of calf thymus poly(A) RNA results in the synthesis of a 120,000-M<sub>r</sub> polypeptide with DNA polymerase activity. This finding opens the way for cloning of the DNA polymerase genes. A panel of monoclonal antibodies against mammalian polymerases  $\alpha$  and  $\beta$  were characterized and evidence was obtained for cross-reactivity of the two enzymes. The antibodies are now being used in studies of DNA polymerase structure. A novel DNA-exonuclease has recently been purified from preparations of helix-destabilizing protein-1 (see previous reports). The enzyme is a 41,000-M<sub>r</sub> polypeptide which releases 5' oligonucleotides from single-stranded DNA substrates but does not degrade double-stranded DNA, closed circular DNA or RNA.

C. (Drs. Edward Kuff and Kira Lueders, and coworkers). This group is studying a family of endogenous retroviral genes related to the mouse intracisternal type-A particle (IAP). There are from 500 to 1000 copies of these genes per haploid genome in Mus musculus and closely related mouse species, whereas species whose progenitors diverged from those of M. Musculus about 4.5 million years ago (Mus cervicolor and Mus caroli) contain only 25 to 50 copies. Multiple individual IAP genes have been cloned from a BALB/c gene library and characterized (see previous reports). In the past year, studies have been extended to the related genes in rats (350 copies), Syrian hamster (800 copies), and other mouse species. The analysis of cloned genes and whole genomic DNA indicates that amplification began between 2.5 and 4.5 million years ago in the lineage of Mus musculus and was probably a relatively recent occurrence in the Syrian hamster line of descent as well. On the other hand, the rat IAP gene family shows a great deal of internal heterogeneity, consistent with an evolutionarily more distant amplification event. The relatively few copies found in Mus cervicolor also seem to be heterogeneous. The data suggest that the IAP gene family has undergone independent amplification events at different times in the evolution of the species studied. This group



is also studying the localization of IAP on specific mouse chromosomes and possible proximities between IAP sequences and other known genes. Thus far, multiple IAP gene copies have been definitively associated with chromosomes 6, 15, and X in *M. musculus*. In addition to the case of the mouse pseudo- $\alpha$ -globin gene described in an earlier report, IAP sequence elements have been found nearby several (but not all) immunoglobulin light chain variable region genes ( $\kappa$  and  $\lambda$ ) and near at least one other endogenous retrovirus-like (VL30) DNA sequence.

#### IV. CELLULAR REGULATION SECTION (Dr. Martin Rosenberg, Chief)

The work of the Section is carried out by two independent groups.

A. (Martin Rosenberg and coworkers). Recombinant DNA techniques have been used to develop a plasmid, phage, and bacterial vector system which allows the isolation, characterization, and comparison of prokaryotic transcriptional regulatory signals. This system has been used to study the efficiencies with which various promoter and terminator signals function *in vivo*. In addition, the system allows selection of mutants in a variety of sites and the precise correlation of the structural alterations with their functional effects. To date the system has been used to introduce and functionally characterize point mutations in several promoter and terminator signals. In each case the effects on signal efficiency have been monitored and related to the structural alteration of the site. This information has led to the development of another vector system which has the potential of (over)producing essentially any protein within the bacterial cell. A strong promoter, which can be regulated effectively in high copy number has been fused adjacent to efficient prokaryotic ribosome binding sites. Coding information for several prokaryotic and eukaryotic gene products has been fused into these systems. In one case we have obtained high level production of the mouse gene for metallothionein and shown this gene product to function in bacteria and protect cells from heavy metal poisoning.

The above system has also been used successfully to overproduce a 97 amino acid polypeptide (cII) encoded by phage  $\lambda$ , which serves as a positive activator of transcription by RNA polymerase. Large amounts of the protein have been obtained and a variety of physical and biochemical studies on this molecule have been initiated. Interactions of this protein with itself, with DNA, and with RNA polymerase are being studied. In addition, 9 single amino acid variants of this protein have been selected by mutation, and similarly overproduced. These variant proteins are now being biochemically characterized.

Previous work demonstrated that under the appropriate conditions prokaryotic genes could be expressed efficiently in eukaryotic cell-free systems. Dr. Rosenberg and his colleagues demonstrated efficient expression of the *E. coli* galactokinase (*galK*) gene directly within mammalian cells. High levels of the bacterial *galK* are produced in a variety of mammalian cells transfected with an SV40-plasmid recombinant vector carrying the *E. coli galK* gene. On this vector, the *galK* coding sequence was inserted downstream from the SV40 early promoter so that the translational start codon for *galK* became the first AUG on the transcript. To ensure proper transcript maturation, SV40 regulatory sequences for RNA splicing and polyadenylation are incorporated beyond the *galK* coding region. Cells transfected with this vector produce large amounts of a new *galK* activity which is similar, by starch gel electrophoresis, to that found in *E. coli*.



The ability to obtain expression of an easily assayable gene product from a structurally defined fusion vector has now permitted studies of: (1) functional complementation by the bacterial enzyme of primary cells isolated from human patients with galK-deficiency galactosemia or other galK-deficient mammalian cells; (2) transcriptional regulation in mammalian cells by fusing various eukaryotic promoters to the galK gene; and (3) translational regulation in mammalian cells by selectively altering the primary structure of the 5' nontranslated portion of the transcript. These vectors have been modified so as to contain a second independent, selectable, assayable marker; the xanthine-guanine phosphoribosyltransferase gene product. Using this gene product as an internal reference, quantitative analyses of various eukaryotic regulatory signals can now be performed.

B. (Dean Hamer and coworkers). The general objective of this group is to understand the regulated expression of animal cell genes. Towards this end they have used recombinant DNA techniques to construct DNA tumor virus hybrids carrying various chromosomal eukaryotic genes. These recombinant molecules have been introduced into cultured mammalian cells by infection, transfection or transformation. Such experiments allow examination of regulatory sequences, such as those required for the heavy metal-inducible transcription of the mouse metallothionein-I gene; overproduction of useful gene products, such as human growth hormone; and analysis of the effects of naturally occurring mutations, such as a deletion that causes a human thalassemia.

#### V. DEVELOPMENTAL BIOCHEMISTRY SECTION SUMMARY (Dr. Igor B. Dawid, Chief)

This Section is composed of three independent groups.

A. (Igor Dawid and coworkers). This group has continued its study of the structure and expression of rRNA genes from Drosophila with the primary aim of understanding the cause for the inactivity of interrupted genes. Run-off transcription in isolated nuclei of Drosophila cells showed that insertions are not transcribed in vitro; thus isolated nuclei preserve in vivo control in this respect. Polymerase loading on regions upstream and downstream of the insertion was measured, leading to the suggestion that interrupted rRNA genes are transcribed up to the point of insertion but not beyond. Preliminary experiments were carried out towards studying rDNA transcription in a reconstituted system. Plasmids containing modified rDNA are being constructed that will allow the assay of transcription of homologous rDNA introduced into cells.

A study of the organization of the genome of Drosophila melanogaster has been carried out in collaboration with M. Gans in Gif-sur-Yvette, France, and P. Wellauer and K. Illmensee in Geneva, Switzerland. The structure of the transposable DNA sequence called the F element has been studied. Three DNA regions containing F elements were isolated from one stock of D. melanogaster and the boundaries of the F elements were sequenced. The corresponding regions were isolated and sequenced from a different stock of D. melanogaster; the F element was absent from these regions. The comparison of the filled and empty sites showed that F element insertion is accompanied by a target site duplication of 8 to 13 base pairs. F elements lack internal repeats, and contain a stretch of about 20 A residues at one end. F elements constitute a novel class of transposable sequence in Drosophila.

A female sterile mutant called *fs(1)h* that leads to homeotic transformations in *Drosophila* is being studied with the aim of isolating the DNA encoding this gene. For this project DNA probes from the chromosomal region of interest have been obtained and a "chromosomal walk" has been initiated. Translocations with breakpoints in or close to the gene have been obtained and will be helpful in identifying the region of interest.

To achieve transformation in *Drosophila* several plasmids have been constructed in which the alcohol dehydrogenase gene has been inserted into transposable elements. These plasmids will be injected into *Drosophila* embryos by collaborating workers in Switzerland. Transformation of mutant flies to alcohol dehydrogenase positives will be sought.

Gene expression during development in *Xenopus laevis* has been studied in several ways. The structure of a repeated DNA sequence that is expressed during development of *Xenopus laevis* has been studied. A conserved repeat unit of about 3.5 kb is flanked by clusters of tandemly repeated sequences with a 180 bp period. To study genes that are first expressed early in development a cDNA library highly enriched for sequences expressed in gastrula but rare or absent in unfertilized eggs has been prepared. A cDNA clone derived from mRNA for the ubiquitous Ca-binding protein calmodulin has been isolated; the sequence of this cDNA has been determined. The sizes of the major calmodulin mRNAs in *Xenopus* have been determined. Genomic sequences containing the calmodulin gene have been isolated from a library of *Xenopus* DNA in lambda phage and are being characterized at present.

B. (Bruce Paterson and coworkers). Using defined, cloned double-stranded cDNA probes for various genes expressed in differentiated chick muscle cell cultures, the corresponding genomic sequences for  $\alpha$  and  $\beta$  actin, vimentin, a major intermediate filament protein, and GAPDH (glyceroldehyde phosphate dehydrogenase) have been isolated.

Preliminary characterization of the actin gene family suggests there are two different  $\alpha$  genes and one  $\beta$ -gene. DNA sequence studies around the 5' proximal intron in the 2  $\alpha$  gene isolates demonstrate complete coding sequence homology yet no homology within the intron. The promoter regions for the different genes are being sequenced in order to pursue promoter regulation studies in eukaryotic vector systems.

In vivo transcription of the vimentin gene, a single copy sequence, yields two distinct mRNA transcripts by Northern analysis. Sequence studies of various vimentin cDNA clones demonstrate the 3' noncoding region of the gene contains 4 polyadenylation sites clustered in pairs approximately 300 base pairs apart. The in vivo mRNA transcripts differ by approximately 300 bp. Furthermore, the cDNA clone containing the pair of poly A sites nearest the 3' end of the coding region hybridizes to both in vivo mRNA transcripts, whereas the cDNA clone containing the more distal polyadenylation sites hybridizes only to the larger mRNA transcript. This suggests that either polyadenylation cluster is used in vivo. The function of this heterogeneity is unclear at present.

The GAP cDNA clone from chicken was used to isolate the homologous gene in yeast. Two of the three different yeast GAP genes were characterized with regard to transcription initiation and termination sites.

C. (Carl Wu and coworkers). The orderly compaction of DNA in nucleosomes along the chromatin fiber is punctuated by highly nuclease-sensitive sites. Such hypersensitive sites have been found in cellular chromatin by mild cleavage of chromatin in isolated nuclei with DNase I, and Southern analysis of the partially cut DNA using cloned hybridization probes. DNaseI hypersensitivity is located at the 5' terminus of several heat-inducible (heat shock) genes in *Drosophila*, and is present during and before induction. Currently the fine structure of the DNaseI hypersensitive site is being studied using DNase I and restriction endonuclease digestion of chromatin and using high resolution Southern blots to analyze the cleavage patterns. Preferentially accessible sites in chromatin such as these may define a state of potential activity for eukaryotic genes, and may function as entry sites to the DNA sequence for RNA polymerase and control factors.

#### VI. MACROMOLECULAR INTERACTIONS SECTION (Dr. Claude B. Klee, Chief)

The section is concerned with the mechanism of the coordinated regulation of cellular processes by  $\text{Ca}^{2+}$  and cAMP. In most, if not all eukaryotic cells, the action of  $\text{Ca}^{2+}$  is modulated by the  $\text{Ca}^{2+}$ -receptor protein, calmodulin. This group previously showed that the sequential binding of  $\text{Ca}^{2+}$  to calmodulin is accompanied by stepwise conformational changes which allow calmodulin to transform a quantitative change in  $\text{Ca}^{2+}$  concentration into qualitatively different cellular responses because different enzymes interact with and are activated by different  $\text{Ca}^{2+}$ -calmodulin conformers. These different  $\text{Ca}^{2+}$ -dependent interactions also allow calmodulin to regulate the  $\text{Ca}^{2+}$  signal kinetically. In addition, two proteins, phosphorylase kinase and calcineurin, exhibit dual  $\text{Ca}^{2+}$  regulation since each contains a  $\text{Ca}^{2+}$ -binding subunit; calmodulin in the case of phosphorylase kinase, calcineurin B in the case of calcineurin. Each interacts with exogenous calmodulin as well. Determination of the amino acid sequence of calcineurin B is almost completed in collaboration with Dr. Cohen and his colleagues. This protein is clearly homologous to calmodulin.

Despite these different modes of interaction, calmodulin cannot interact simultaneously with more than one target protein; no ternary complex including calmodulin has yet been detected. Among the calmodulin fragments obtained by limited proteolysis, peptide 78-148 is the only one which has preserved both its  $\text{Ca}^{2+}$  binding property and ability to interact with two different target proteins, phosphorylase kinase and cyclic nucleotide phosphodiesterase. This peptide can activate phosphorylase kinase but inhibits the activation of phosphodiesterase by calmodulin. Different enzymes may therefore recognize the same calmodulin domain but have different requirements for activation.

The calmodulin binding proteins including cyclic nucleotide phosphodiesterase, calcineurin, myosin kinase and  $(\text{Ca}^{2+} + \text{Mg}^{2+})$  plasma membrane ATPase, become activated upon limited proteolysis and lose their  $\text{Ca}^{2+}$  dependence. They are all therefore composed of two domains, a catalytic domain and a regulatory, calmodulin binding domain, which exerts an inhibitory role in the absence of calmodulin. Preliminary experiments suggest that these inhibitory domains contain a common calmodulin binding site. Current work in the laboratory is directed towards the characterization and isolation of the interacting sites on calmodulin and its target proteins. These studies will lead to a better understanding of the unique property of calmodulin, its ability to interact with so many different proteins.



Another aspect of  $\text{Ca}^{2+}$ -calmodulin regulation of cellular processes is its coupling with that of cAMP. Most calmodulin regulated enzymes are also regulated by cAMP dependent phosphorylations which enhance or inhibit their stimulation by calmodulin. Conversely, calmodulin and  $\text{Ca}^{2+}$  modify cAMP-dependent responses directly at the level of cyclic nucleotide metabolizing enzymes. In collaboration with Dr. Cohen and his colleagues, we have recently isolated a  $\text{Ca}^{2+}$ -calmodulin-dependent protein phosphatase which copurifies with calcineurin. This phosphatase exhibits strict substrate specificity. It dephosphorylates phosphorylase kinase inhibitor -1 and myosin light chains in skeletal muscle; in brain it dephosphorylates the regulatory subunit of protein kinase (in the presence of cAMP) and may thereby regulate cAMP-dependent phosphorylation. Thus, another link between the two second messengers has been detected in the calmodulin regulation of cAMP-dependent protein kinase.

## VII. NUCLEIC ACID ENZYMOLOGY SECTION (Dr. Maxine Singer, Chief)

There are two research interests being studied by this group. First, they are studying the relation between the genomes of simian virus 40 (SV40) and the African Green Monkey (AGM). During a productive permissive infection of AGM cells by SV40 the virus coopts the cell's resources for its own reproduction. The virus is not passive in the process: after the host's RNA polymerase, ribosomes, etc., synthesize the viral encoded T-antigen, this protein in turn stimulates the synthesis of cell-encoded enzymes required for DNA synthesis and cellular DNA synthesis itself. These observations suggest a significant relation between the structures of the SV40 and AGM genomes. A library of the AGM genome in  $\lambda$  bacteriophage yielded three different AGM DNA segments that are homologous, in part, to the region around the SV40 origin of replication. Each homologous segment is a few hundred base pairs in length, and the sequences were determined earlier. Experiments with one of the AGM segments have been carried out in collaboration with Paul Berg's laboratory using SV40-based vectors constructed there. A second segment is being studied here.

In brief, these studies involve construction of recombinant vectors containing an E. coli gene whose expression can be detected in animal cells, an E. coli plasmid vector segment that allows amplification of the molecules in bacteria prior to transfection into animal cells, sequences that provide for transcription termination and splicing of messenger RNA molecules, and appropriately placed regions where different DNA segments can be placed at will. The results indicate that the monkey DNA segments do not replace the SV40 control region in support of DNA replication. They do, however, provide transcriptional start sites at which the synthesis of messenger RNA begins. Thus, these constructions permit expression of the E. coli gene in monkey and mouse cells. However, the monkey segments do not contain all the elements necessary for transcription. A special region of the SV40 genome, by itself insufficient for transcription, must be included at some position in the vector; the position and orientation is irrelevant. This SV40 sequence is known as the "72 base pair repeat." Thus these results indicate that the 72 base pair repeat enhances transcription, so long as appropriate transcriptional start sites are available. The start sites within the monkey sequences are being mapped by the S1 and primer extension methods in order to study the specific sites.

The entire 17 kilobase pair cloned monkey DNA region surrounding one of the regions homologous to the SV40 origin region is being characterized. The

results show that the short origin-like segment contains a DNase I hypersensitive site in monkey chromatin and is homologous to cellular RNA. The 450 base long region is located in a genomic region that contains multiple and different interspersed repeated sequences representing at least three different families of such sequences typical of the monkey genome.

The second project pursued by this group is the study of highly repeated sequences in the genome of the African green monkey. Both satellites and interspersed repeated sequences are investigated. A new monkey satellite with a ten base pair repeat length was discovered, and the sequence of cloned portions were determined. This "deca-satellite" joins directly, in centromeric regions, to the  $\alpha$ -satellite. Remarkably, deca-satellite is highly polymorphic; the pattern of restriction endonuclease bands varies from one individual monkey to another in striking fashion. An interspersed repetitive unit about 6 kilobase pairs in length that occurs thousands of times in the monkey genome and also in other primates is being characterized. One copy interrupts a cloned segment of monkey  $\alpha$ -satellite DNA, suggesting that the element may be a moveable element. Three short DNA segments that occur hundreds of thousands of times interspersed in the monkey genome are also being studied. One of these is the well-known primate element called Alu. Alu has been proposed by many to be a moveable element. This group obtained evidence directly in favor of this hypothesis by demonstrating that a copy of Alu interrupting  $\alpha$ -satellite is flanked by 13 base pair direct repeats of a segment that occurs only once in the normal satellite sequence. The other two elements were discovered in this laboratory during the past year. It has been shown that one of them is flanked by a duplication of the target site suggesting that it is moveable. Both newly discovered elements hybridize with cellular RNA. The work on the deca-satellite and the interspersed repeated elements together emphasize a growing understanding of the dynamic nature of primate genomes.

#### VIII. PROTEIN CHEMISTRY SECTION (E. A. Peterson, Chief)

The work of this section is carried out by four independent groups.

A. (O. Wesley McBride and coworkers). Analysis of somatic cell hybrids segregating human chromosomes was used to localize human *onc* gene analogues, immunoglobulin genes, and Ig pseudogenes to specific human chromosomes. Several large series of independent hybrid cell lines were isolated and the human chromosome content determined by isoenzyme analyses and karyotyping. Parental donors were normal cells, fibroblast lines containing specific, well-characterized translocations or deletions, and peripheral leukocytes containing the Ph chromosome. Human donor lines were chosen for the presence of appropriate translocations permitting regional gene mapping, selective retention and loss of appropriate translocation fragments, and identification of reciprocal chromosome translocation products and their normal homologues by isoenzyme analysis. In collaboration with Dr. David Swan, DNA was isolated from each cell line and analyzed by Southern blotting analysis using isotopically labeled cloned DNA probes. Studies in collaboration with Dr. Philip Leder and colleagues resulted in assignment of constant region kappa ( $C_K$ ) and lambda ( $C_\lambda$ ) Ig genes to chromosomes 2 and 22, respectively. The entire group of heavy chain genes ( $V_H$ ,  $D_H$ ,  $J_H$ , and  $C_H$ ) were regionally assigned to a single band, 14q32. Immunoglobulin pseudogenes were assigned to chromosomes 9( $\psi_2$ ), 14q32( $\psi_1$ ), 15( $\psi$ ), and 18( $\lambda\psi$ ). Two of these pseudogenes exhibit interesting properties indicating an RNA-intermediate in their formation as well as properties of a transposable



element. In collaboration with Dr. Stuart Aaronson and colleagues, human cellular onc gene analogues were assigned to chromosomes 22(c-sis), 8(c-mos), 6(c-myb), and 11(bcl or c-bas). The three Ig bearing chromosomes are known to be involved in specific reciprocal translocations with chromosome 8 in B cell neoplasms thereby suggesting a possible mechanism for the origin of these lymphomas. Mapping of human onc gene analogues will greatly facilitate analysis of the role of onc genes in human cancer.

Work is progressing on the isolation of the human tk gene from a recombinant DNA library containing the gene in a mouse background using plaque hybridization with a repetitive human DNA probe. Extensively characterized transformants resulting from chromosome-mediated gene transfer are being used by Dr. David Housman at MIT to analyze the distribution of a family of dispersed repetitive DNA sequences on the X chromosome and as a resource to clone the human gfpd gene.

B. (Michael Mage and colleagues). One goal of this project is the development of methods for the specific isolation of the antigen-reactive cells that are involved in cellular immune reactions to alloantigens. Another goal is to understand better the differentiation of cytotoxic effector cells (CTL) from precursors in the thymus. A third goal is to understand the molecular reactions between immune cells in T cell subpopulations and antigens bound to target cell surfaces that lead to in vivo cellular immune phenomena such as graft rejection and the graft-versus-host reaction.

Using highly purified populations of  $\text{Lyt2}^+$  and  $\text{Lyt2}^-$  T cells obtained by a nonlytic separation technique previously developed, this group has found that help by  $\text{Lyt2}^-$  helper cells for CTL generation in mixed lymphocyte culture is not antigen-specific, a finding compatible with their postulated role of providing the lymphokine IL2. Also, with the relative numbers of  $\text{Lyt2}^+$  and  $\text{Lyt2}^-$  cells present in unimmunized mice, the amount of CTL activity that can be generated is not limited by the number of CTL precursors but by the amount of help that is provided by the  $\text{Lyt2}^-$  helper cells. Other recent experiments have shown that macrophage-like cells are required for such help. When these cells were removed by preincubation in uncoated tissue culture dishes (to which they adhered) prior to cell separation procedures, little or no CTL activity was generated, even when  $\text{Lyt2}^+$  helper cells were added. However, the addition of IL2 restored the ability of the mixed culture to generate CTL activity, indicating that the function of the  $\text{Lyt2}^-$  cells can be served by the addition of their putative product, the lymphokine IL2.

C. (Warren Evans and colleagues). Studies by this group are aimed at identifying the regulatory factors that control the induction of synthesis of specific proteins associated with the differentiation of bone marrow leukocytes. A major goal of this research has been attained with the development of a direct assay for granulocyte differentiation proteins. This assay utilizes high pressure liquid chromatography (HPLC) to screen extracts of leukocytes for a variety of proteins that appear in the cell membrane and secondary granules only during differentiation. It is rapid (40 minutes) and requires only  $10^6$  cells, about the number of granulocytes in 0.5 ml of blood. Studies using leukemic cells from the guinea pig model for human chronic myelogenous leukemia (CML) developed in this laboratory indicate that this HPLC assay will provide a valuable new tool for phenotypically characterizing leukemic granulocytes at various stages of this disease and for monitoring the effectiveness of therapeutic regimens aimed at inducing maturation of leukemic cells.

Another major goal has been achieved in the development of a cell culture system that permits the study of the regulation of the maturation of normal and leukemic immature granulocytes at the morphological and biochemical levels simultaneously. This has been accomplished by the selection of culture conditions that permit granulocyte differentiation to be studied in the absence of cell proliferation. Unlike the widely used agar colony assay, this new system makes it possible to distinguish readily between factors that promote proliferation and those that promote differentiation. The mature granulocytes formed in this system have a normal morphology, and HPLC assays of cell extracts show that morphological maturation of immature granulocytes *in vitro* is accompanied by the induction of synthesis of all of the major differentiation markers detected by the HPLC assay in mature granulocytes formed *in vivo*. Humoral factors that control the induction of differentiation proteins in normal immature granulocytes are being investigated, as well as the biochemical mechanisms of arrested maturation of leukemic granulocytes, with the aim of determining the potential reversibility of the cell maturation defects in leukemia.

The transplantable, granulocytic leukemia (GL-13-BC) previously developed in this laboratory in inbred strain 13 guinea pigs is an attractive model for human CML. The model has been standardized, using a subcutaneous route of injection to give a highly reproducible clinical course of the disease and is presently being used to develop new therapeutic regimens for delaying or preventing the onset of blast crisis, a stage of the disease that is almost always fatal in cases of human CML.

D. (E. A. Peterson). The narrow-range carboxymethyl dextrans (CM-Ds) prepared by fractionating the heterogeneous products obtained from the reaction of alkaline dextran with chloroacetate have been tested by applying them to the displacement chromatography of proteins known to have similar isoelectric points.  $\beta$ -Lactoglobulins A and B are genetic variants that differ in isoelectric point by about 0.1 pH unit. They are difficult to separate by elution chromatography though it has been done. They were easily separated by displacement chromatography on DEAE-cellulose, using a narrow-range CM-D, nearly all of which emerged within and between the two peaks. This preparation was selected by using the pellet volume assay (report for 79-80) to identify the most effective CM-D in a preceding experiment employing five unfractionated preparations that covered a wide range of affinity.

Commercial ovalbumin (99%, on the basis of electrophoretic analysis) proved to comprise several more components separable by displacement chromatography than the three forms containing 0, 1, and 2 phosphate groups, respectively, that were initially expected. However, heterogeneity based on known differences in carbohydrate content, as well as dimer formation, may account for the multiplicity observed.

A protein can be separated from the CM-D that accompanies it in the effluent fractions by lowering the pH to the vicinity of the protein's isoelectric point and passing the fraction through a column of DEAE-cellulose equilibrated with  $\text{NaH}_2\text{PO}_4$ . The protein passes through unadsorbed, leaving the CM-D bound to the column. Preliminary experiments indicate that serial application of selected fraction to the same column may be used to reclaim spacer CM-Ds for re-use, while collecting the desired protein products.

The preparation of narrow-range CM-Ds has been simplified and the products improved by preparing the full range of CM-Ds from one reaction mixture. Portions are removed continuously during the reaction period and collected in only four fractions prior to purification and fractionation into an array of products having progressively higher affinity.

#### IX. OFFICE OF THE CHIEF

Dr. Cecil Fox is detailed to the Armed Forces Institute of Pathology, where he carries out his research. The goals of this program are to use biological characteristics of tumor cells as a means for improving grading of human cancer through use of more objective parameters. The object of grading tumors is to estimate the potential of the tumor for growth, invasion, and metastasis which are important factors to clinicians in planning therapy. Tumor cell attachment to new substrates is being compared to normal cell attachment using reflection contrast microscopy. A major characteristic of cancer cells, release from density dependent growth control, is also being studied as is the role of lamellar cytoplasm in tumor cell growth. A long-term study of the pathology and dynamics of infections in immune compromised patients is in progress. A preliminary investigation of the histiogenesis of lepromatous and histoid leprosy as a model of mesenchymal dysplasia has been started. One project characterizes human cancer cell clonogenic populations in archival tissues and with needle aspiration cytology. A study of endometrial epithelium and its growth dynamics in relation to endometrial hyperplasia is now underway, and a separate project on the dynamics of cellular response to cytostatic agents in head and neck cancer is in progress.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 00333-19 LB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Biochemical Basis for Defective Differentiation in Granulocytic Leukemia		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
P.I.  Others	W.H. Evans  E.A. Peterson  S. Wilson M. Mage V. Alvarez	Research Chemist      LB NCI Research Chemist      LB NCI Chief, Protein Chemistry Section Biologist              LB NCI Immunochemist        LB NCI Expert                  LB NCI
COOPERATING UNITS (if any)  Hematology, Oncology Section, Walter Reed Army Medical Center		
LAB/BRANCH  Laboratory of Biochemistry		
SECTION  Protein Chemistry Section		
INSTITUTE AND LOCATION  NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  3	PROFESSIONAL:  2	OTHER:  1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>The main thrust of this work is to develop biochemical methods for the early diagnosis of <u>granulocytic leukemia</u> and methods for inducing leukemic cells to develop some or all of their functional properties as a means of partially or completely restoring host defense mechanisms in leukemia patients. Work is first aimed at establishing which of the many biochemical steps involved in normal <u>granulocyte differentiation</u> are controlled by humoral regulators. The results will be compared with those obtained from similar studies on leukemic cells at corresponding stages of maturity in order to determine the nature and potential reversibility of the arrested differentiation steps. Biochemical analyses are carried out on mature and immature granulocytes isolated from <u>blood and bone marrow</u> and the effects of external cell regulators on <u>granulocyte differentiation</u>, as measured by changes in the synthesis of specific cellular components, are studied in a defined culture system previously developed in this laboratory.</p>		



Methods Employed: Leukocytes are isolated from bone marrow, blood and inflammatory exudates by a variety of methods. Subcellular fractions are prepared by differential centrifugation. Proteins extracted from leukocytes and serum proteins are fractionated by column chromatography, high performance liquid chromatography (HPLC), and gel electrophoresis. Glycolipids are separated by thin layer chromatography and analyzed by gas-liquid chromatography and mass spectrometry. Immunological reagents, including fluorescent antibodies, are prepared against granulocyte proteins and used to follow their formation during granulocyte maturation. Stationary tissue culture methods are employed to study the synthesis of specific proteins from radioactive precursors. The distribution of radioactive proteins in electrophoretic gels is determined by slicing the gels in a gel fractionator and counting the slices in a liquid scintillation counter. Densitometric patterns of gels stained for glycoproteins are obtained by scanning the gels in a spectrophotometer equipped with a gel scanner accessory.

Major Findings:

A. Development of a direct, rapid assay for granulocyte differentiation proteins.

One of the important goals of our research was achieved this year with the development of a direct and rapid biochemical assay for protein markers of granulocyte differentiation. Most assays for granulocyte differentiation currently available are either non-specific or involve time consuming and laborious procedures. Previously we showed that many of the markers of granulocyte differentiation were glycoproteins that could be extracted from these cells using various detergent solutions. We found, however, that most of these proteins could not be readily separated by standard chromatographic procedures. The fact that many of these proteins appeared to have hydrophobic properties suggested to us that certain recently developed HPLC techniques for separating hydrophobic proteins might be useful in separating granulocyte differentiation proteins. Our studies thus far indicate that reverse phase HPLC is a powerful new tool for analyzing leukocyte extracts for such proteins. With this method it is possible to rapidly and simultaneously screen cells for a variety of cell membrane and secondary granule differentiation markers. This assay takes only 40 minutes to perform and requires only a small number of granulocytes (about  $1 \times 10^6$  cells or the number of granulocytes in about 0.5 ml of blood). Other studies using leukemic granulocytes from our recently developed guinea pig leukemia model for human CML indicate that this new HPLC assay will also provide a valuable new tool for phenotypically characterizing leukemic granulocytes during various stages of the disease and should also be useful in monitoring the effectiveness of therapy aimed at inducing leukemic cells to mature. As indicated below we are also finding this new technique useful in studies of the biochemical maturation of normal and leukemic immature granulocytes in vitro.



B. Studies of the induction of maturation in normal and leukemic immature granulocytes.

Another major research goal attained this year was the development of a cell culture method that permits us to study the regulation of normal and leukemic immature granulocyte maturation at the morphological and biochemical levels. Success in this area was partly due to the fact that our new HPLC assay for differentiation is far more sensitive and much more rapid than our previous assays and this permits us to study many more variables affecting granulocyte differentiation in each experiment.

At present the most widely used assay for regulators of normal granulocyte production employs semi-solid agar cultures in which the number of cell colonies formed from granulocyte precursors is used as an indicator for detecting regulators of granulocyte production. While this technique has proven very useful in the study of the biological aspects of the regulation of granulocyte production, technical problems with harvesting cells reproducibly from the agar cultures in sufficient quantity for biochemical analyses have made biochemical studies of granulocyte maturation by this method very difficult. Furthermore, because the formation of granulocyte colonies requires the continual presence of growth promoting factors it is not possible with this assay to distinguish between a proliferation-promoting and a differentiation inducing regulator without a detailed and laborious morphological analysis of each colony counted.

For our assay system we first isolate large numbers of immature granulocytes from normal guinea pig bone marrow using a ficoll density centrifugation procedure previously developed in this laboratory. The immature granulocytes are placed in stationary liquid cultures containing RPMI-1640 medium with various inducing agents and incubated for periods up to one week at 37° in an atmosphere of 5% CO<sub>2</sub> and 95% air. With this technique we find it is possible to readily harvest cells for both morphological and biochemical analyses. Furthermore, our culture conditions are chosen such that differentiation can be studied in the absence of cell proliferation making it possible to distinguish between proliferation and differentiation promoting factors. Our results with this system indicate that when normal immature leukocytes are incubated for 6 days in the presence of 10% dialyzed normal guinea pig serum, these cells are almost all converted to mature granulocytes that have a normal morphology. The HPLC patterns of proteins in cell extracts analyzed before and after cell culture show that the morphological maturation of immature granulocytes in vitro is accompanied by the induction of synthesis of all the major differentiation markers detected by this assay in mature granulocytes formed in vivo. In confirmation of results obtained with the agar culture assay, our studies show that immature granulocytes have the potential to form either mature granulocytes or macrophages, depending on the incubation conditions. The ratio of mature granulocytes to macrophages derived from immature granulocytes appears to be directly related to the level of normal serum proteins present in the medium. Thus at 10% serum mostly mature granulocytes are present after 6 days, whereas below 1% serum mostly macrophages are found in the culture.

This finding suggests that the direction of granulocyte differentiation could be controlled either by the level of non-specific protein in the medium or by a specific factor present in normal serum that is required for the conversion of immature cells to mature granulocytes. Our preliminary results with various protein fractions isolated from normal serum by column chromatography favor the latter possibility.

Using this culture system we are also studying immature leukemic cells from the blast crisis phase of our guinea pig model for human CML for their potential to be induced to mature in vitro. This approach is based on the recent recognition that the leukemic process does not always involve genetic changes that preclude further differentiation and that some leukemic cell populations can be induced to differentiate in vitro if exposed to the proper external stimuli. Our results show that leukemic cells from the blast crisis stage fail to mature under conditions described above which support the maturation of normal immature cells. We are currently investigating a wide variety of other factors which might stimulate leukemic cell maturation in vitro. It will also be of interest to compare blast crisis cells with immature cells from the earlier differentiating phase of this leukemia to determine how much the latter cells differ from the former cells with respect to their degree of competence for induction of maturation at the morphological and biochemical levels.

#### C. Continuous growth of guinea pig leukemic granulocytes in cell culture.

During the past year we have successfully established a continuous cell culture of leukemic leukocytes from the GL-13 guinea pig leukemia previously developed in this laboratory. We find that these cells will grow continuously in stationary cultures containing RPMI-1640 medium fortified with 10% fetal calf serum and in an atmosphere of 5% CO<sub>2</sub> and 95% air. Cell lines from both the early differentiating and blast crisis stages of this disease have been established in culture and both have a doubling time of approximately 36 hours. Under these conditions both cell lines appear similar morphologically and show no evidence of cell maturation. Dr. Howard Terebello at Walter Reed Hospital is using these cultures to study the effects of a variety of therapeutic agents used in human leukemia on the proliferation and maturation of these cells. Any therapeutic agent or combination of agents that arrest growth and/or induce cell maturation of the leukemic cells in vitro will be tested in vivo in the guinea pig leukemic model.

Significance to Cancer Research: (Objective 2, Approach 3). Granulocytic leukocytes are produced in the bone marrow by a complex, multistage, process of cell differentiation whereby proliferating, nonphagocytic, precursor cells are converted to nonproliferating phagocytic cells that play a crucial role in the body's defense against microbial invasion. Granulocytic leukemias can be thought of as diseases in which this program for differentiation is arrested at various stages, resulting in the overproliferation of immature cells lacking, in varying degrees, the capacity to carry out their normal function in the body's defense against infections. At present, the mechanisms involved in the initiation and modulation of the various steps in the differentiation program are poorly understood. Our research is aimed at clarifying the molecular

processes that control the appearance of specific subcellular components during granulocyte differentiation. Such information should be useful in developing therapeutic approaches for reversing the arrested differentiation of leukemic cells.

Proposed Course of Research: The rate of differentiation of granulocytic leukocytes in the bone marrow is regulated by a feedback control mechanism which responds to the rate of destruction of these cells in the peripheral tissues of the body. Feedback information could be of a positive (stimulator) or negative (inhibitor) type transmitted by humoral and/or cellular factors, but at present the nature of these factors is unknown. The humoral hypothesis for regulation will be examined by studying the effect of normal and inflammatory sera on granule-specific protein synthesis by granulocyte precursors in vitro. This approach is analogous to the use of hemoglobin as a marker for erythrocyte differentiation in assays for erythropoietin. Unlike erythrocytes, which contain only one major specific protein, granulocytes contain many potential marker proteins in their granules. Looking at this problem from a broader perspective, it is conceivable that the system we are developing for the study of the coordinated regulation of macromolecular synthesis associated with secondary granule formation in myelocytes could serve as a useful model for studies of coordinate gene expression in mammalian cells, just as the study of hemoglobin formation in erythrocyte differentiation has provided much information about the regulation of single genes. Our research plans are as follows:

- (1) To isolate, purify and eventually test in vivo any humoral regulators of granulocyte differentiation detected by our newly developed combined morphological and biochemical assay for granulocyte differentiation in vitro.
- (2) To use HPLC as a method for phenotypically characterizing the degree of differentiation of purified populations of normal and leukemic granulocytes from guinea pigs and eventually humans. This rapid screening method might be useful in classifying or staging AML and CML patients for various types of therapy.
- (3) To investigate various potential agents for inducing leukemic cells to mature either partially or completely in vitro. Such agents could serve as attractive adjuncts, or in some cases, as alternatives to cytotoxic therapy, for they would inhibit the expansion of the leukemic population while leaving normal granulocytes unharmed.
- (4) The standardized guinea pig model for human CML will be used to test the effectiveness of conventional and potentially new therapeutic regimens for delaying or preventing the onset of blast crisis and in increasing the survival of leukemic guinea pigs. In this connection, studies in collaboration with Dr. Kenneth Foon are underway to develop monoclonal antibodies against the guinea pig leukemic cells for the purpose of testing the efficiency of using such antibodies, either alone or as conjugates with various therapeutic agents (drugs, toxins, isotopes), for eradicating leukemic cells in vivo or from syngeneic bone marrow preparations to be used as grafts for repopulating irradiated leukemic hosts.

Publications:

Moore, D.E., Evans, W.H. and Mage, M.G.: A Deletion in chromosome #1 in cells of a transplantable granulocytic leukemia (GL-13) in guinea pigs. J. Natl. Cancer Inst., in press, 1982.

Appendix: NIH Contract No. 1-60-25423

Funding: General NCI Contract for FCRC - No individual project funding breakdown available.

Man Years Purchased: 1

Major FindingsStandardization of the GL-13-BC Leukemia

The transplantable granulocytic leukemia (GL-13-BC) in inbred strain 13 guinea pigs previously developed under this contract has been shown to have many characteristics that make it the most attractive model for human CML now available. Although this leukemia has been maintained by intraperitoneal passage of the leukemic cells for over 100 transplant generations our more recent studies indicate that the subcutaneous route of transplantation is more suitable for experimental studies since the clinical course of the disease is much more consistent and reproducible. Under standardized conditions, guinea pigs injected subcutaneously with  $3.0 \times 10^6$  leukemic cells uniformly develop a hematological leukemia within 20-23 days after injection. This stage of the disease resembles the chronic phase of human CML and is accompanied by the appearance of a tumor nodule at the site of injection. The blood leukocyte count rises sharply 24-28 days after injection and a blast crisis similar to that seen in human CML develops. Shortly thereafter the leukemic guinea pigs die with a mean survival time of 30 days (range 25-32 days). (A paper describing these studies has been submitted for publication). This model is now being used to develop therapeutic methods for delaying or preventing the onset of blast crisis, a stage of the disease that is almost always fatal in cases of human CML.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 00366-12 LB																				
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>																						
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Biosynthesis and Assembly of Intracellular Components</p>																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																						
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 30%;">E. L. Kuff</td> <td style="width: 40%;">Chief, Biosynthesis Section</td> <td style="width: 20%;">LB NCI</td> </tr> <tr> <td></td> <td>K. K. Lueders</td> <td>Chemist</td> <td>LB NCI</td> </tr> <tr> <td>OTHER:</td> <td>R. Callahan</td> <td>Microbiologist</td> <td>LVC NCI</td> </tr> <tr> <td></td> <td>P. Leder</td> <td>Chief, Laboratory of Molecular Genetics</td> <td>LMG NICHD</td> </tr> <tr> <td></td> <td>A. Leder</td> <td>Biochemist</td> <td>LMG NICHD</td> </tr> </table>			PI:	E. L. Kuff	Chief, Biosynthesis Section	LB NCI		K. K. Lueders	Chemist	LB NCI	OTHER:	R. Callahan	Microbiologist	LVC NCI		P. Leder	Chief, Laboratory of Molecular Genetics	LMG NICHD		A. Leder	Biochemist	LMG NICHD
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	P. Leder	Chief, Laboratory of Molecular Genetics	LMG NICHD																			
	A. Leder	Biochemist	LMG NICHD																			
COOPERATING UNITS (if any) <p style="text-align: center;">Laboratory of Viral Carcinogenesis, NCI          Laboratory of Molecular Genetics, NICHD</p>																						
LAB/BRANCH <p style="text-align: center;">Laboratory of Biochemistry</p>																						
SECTION <p style="text-align: center;">Biosynthesis Section</p>																						
INSTITUTE AND LOCATION <p style="text-align: center;">DCBD, NCI, NIH, Bethesda, Maryland 20205</p>																						
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">TOTAL MANYEARS:</td> <td style="width: 33%;">PROFESSIONAL:</td> <td style="width: 33%;">OTHER:</td> </tr> <tr> <td style="text-align: center;">4.5</td> <td style="text-align: center;">2.5</td> <td style="text-align: center;">2.0</td> </tr> </table>			TOTAL MANYEARS:	PROFESSIONAL:	OTHER:	4.5	2.5	2.0														
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CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <span><input type="checkbox"/> (a) HUMAN SUBJECTS</span> <span><input type="checkbox"/> (b) HUMAN TISSUES</span> <span><input checked="" type="checkbox"/> (c) NEITHER</span> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <span><input type="checkbox"/> (a1) MINORS</span> <span><input type="checkbox"/> (a2) INTERVIEWS</span> </div>																						
SUMMARY OF WORK (200 words or less - underline keywords)  <p>         We are studying murine intracisternal A-particles as a model for the <u>evolution</u>  <u>and expression of integrated reiterated viral genes</u>. Multiple individual <u>A-</u>  <u>particle genes</u> have been isolated from mouse DNA using <u>recombinant DNA tech-</u>  <u>niques</u>. These genes number 1000-2000 copies per cell in <u>Mus musculus</u> and          closely related species, but only 50 copies are found in the DNA of more dis-          tant members of this genus. Rat and <u>Syrian hamster</u> DNA contain about 700 and          1600 copies per cell of IAP-related genetic units, respectively; and individual          genes have been isolated from gene libraries of both these animals. The <u>rat</u>  <u>gene family</u> shows a much higher degree of internal divergence than do those of          the <u>mouse</u> and <u>Syrian hamster</u>. Heteroduplex analysis and melting data suggest          that the <u>IAP gene family</u> has <u>undergone independent amplification events</u> at          different times in the evolution of these rodent species.       </p>																						



## Project Description:

Objectives: To study the mechanisms for coordinately regulating the synthesis of individual macromolecules and their assembly into complex intracellular components (organelles and viruses); specifically, to study the regulation of endogenous viral gene expression in relationship to normal development and neoplastic transformation.

Methods Employed: Culture of animal and bacterial cells; recombination and cloning of specific eukaryotic and viral DNA sequences in plasmids, lambda phage and retrovirus vectors; analysis of DNA components by restriction endonuclease cleavage, electrophoresis, and blot hybridization; chromosomal localization of specific DNA sequences by *in situ* hybridization; electron microscopy of DNA heteroduplexes and DNA:RNA hybrid molecules; nucleotide sequencing by procedure of Maxim and Gilbert; chromatographic and electrophoretic analysis of cellular and viral proteins; radio-immunoassay and immunoprecipitation of specific antigens; immunofluorescent staining.

Major Findings: Previous studies in this project have established that intracisternal A-particle (IAP) genes make up an extensively reiterated family of retrovirus-like elements in the cellular DNA of the Mus musculus. The basic genetic unit is 7.3 Kb long, colinear with 35S A-particle RNA, and contains terminal repeat sequences (TRS's) of about 350 base pairs. Genes containing deletions have also been observed. Sequences homologous to the IAP genes of the mouse have been identified in the DNA of other rodent genera, and endogenous IAP sequences in at least one Asian mouse species Mus cervicolor have been shown to participate in the formation of an infectious extracellular retrovirus. IAP sequences have been found to bracket a previously recognized pseudo- $\alpha$ -globin gene ( $\alpha\psi 3$ ) in the DNA of BALB/c mice (work with A. Leder and P. Leder). This spatial relationship is interesting because some type of retroviral intervention had been suggested to account for the fact that  $\alpha\psi 3$  lacks the intervening sequences characteristic of functional  $\alpha$ -globin genes and has been translocated to a different chromosome from the main  $\alpha$ -globin gene cluster. Recent findings are as follows:

### 1. Comparative studies

- a. Earlier we showed that IAP sequences are fully represented in the DNA of two Asian mouse species M. cervicolor and M. caroli, although in copy numbers of about 25 per haploid genome rather than the 500-1000 characteristic of M. musculus. The lineage of these Asian mice is thought to have diverged from that of M. musculus some 4.5 million years (Myr) ago. We have now compared the IAP sequences in a number of more closely related mouse species (M. musculus, domesticus, mollosinus, castaneus, hortulanus, and spretus) considered to have diverged within the last 1 to 2.5 Myr. IAP sequences are abundant in all of the species, although individual species show characteristic differences in the relative proportions of particular restriction site variants. All in all, the data suggest that an amplification of IAP genes began sometime between 2.5 and 4.5 Myr ago and continued independently in the more recently emerging species. Amplification of these genes may still be in progress, facilitated by the known IAP expression in early mouse embryos.

- b. In the past year, genetic elements related to mouse IAP genes have been isolated as recombinants from rat and Syrian hamster gene libraries. Heteroduplex analysis shows that the rat and hamster elements have the same basic linear arrangement as the mouse genes; however, the regions of homology with mouse genes show multiple interruptions indicative of highly divergent segments within the rat and hamster sequences. The rat genome (Sprague-Dawley) contains about 350 copies of IAP-related elements and this gene family is itself internally heterogeneous as revealed both by heteroduplex analysis and melting studies with cloned rat genes. We believe that amplification of the IAP sequences in the rat occurred independently of that in the mouse and most likely at an earlier time. Syrian hamster DNA contains about 800 copies of IAP-related sequences per haploid genome. While these sequences are highly divergent from the mouse genes (as expected from the evolutionary distance between these two rodent families), they appear to be internally homogeneous. It would appear that amplification of IAP genes in this species has occurred relatively recently and independently of the process in the mouse.
  - c. In collaboration with Dr. R. Callahan we have defined the genetic relationship between the IAP from the Mus musculus and the extracellular retrovirus (M432) from M. cervicolor. The major homology region of 3.7 Kb begins near the 3' end of the M432 gag gene, spans the pol gene, and ends in the env gene. We have now isolated M432 virus-related sequences from M. cervicolor genomic DNA making possible a comparative analysis of molecular clones of IAP and M432-related sequences and their organization in this mouse species.
2. Spatial relationships between IAP sequences and other mouse genes  
 Since integrated proviruses can have effects on nearby genes, we have been testing for proximity of IAP sequences to other known genetic elements. In addition to the case of the mouse pseudo- $\alpha$ -globin gene mentioned above, we have found close spatial relationships between IAP elements and (a) other endogenous virus-like (VL30) DNA sequences and (b) several immunoglobulin light chain variable region genes ( $\kappa$  and  $\lambda$ ). Four of five IAP genes found in association with other known mouse genes contained deletions.
  3. Chromosomal localization  
 We are studying the distribution of IAP sequences on individual mouse chromosomes, using both in situ hybridization of mouse embryo mitotic spreads and restriction analysis of DNA from somatic cell hybrids (mouse X Chinese hamster) containing limited numbers of mouse chromosomes. IAP genes are found on most if not all mouse chromosomes. Thus far multiple copies have been definitively associated with chromosomes 6, 15, and X.

#### Proposed Course of Research:

We will continue to investigate the possible effects of IAP genes on the structure, position and activity of other genetic elements. The comparative study of

IAP-related sequences in other species will be continued to elucidate the phenomenon of periodic gene amplification. In view of the invariant expression of IAPs in mouse myeloma cells, we plan to study the possible role of the genetic components in the events leading to B-cell transformation.

#### Significance for Cancer Research:

IAP-related genes are by far the most abundant endogenous retroviral element presently recognized in the mouse and Syrian hamster. Through unprogrammed recombinational events such as transposition or new proviral insertions, they may modify the structure and/or activity of cellular structural genes, including oncogenes.

#### Publications:

Callahan, R., Kuff, E.L., Lueders, K.K., and Birkenmeier, E.: Genetic relationship between the Mus cervicolor M432 retrovirus and the Mus musculus intracisternal type A particle. J. Virol. 40: 901-911, 1981.

Lueders, K.K. and Kuff, E.L.: Sequences homologous to retrovirus-like genes of the mouse are present in multiple copies in the Syrian hamster genome. Nucleic Acids Res. 9: 5917-5930, 1981.

Lueders, K.K., Leder, A., Leder, P., and Kuff, E.L.: Association between a transposed  $\alpha$ -globin pseudogene and retrovirus-like elements in the BALB/c mouse genome. Nature 295: 426-428, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 00375-20 LB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Homogeneity and Structure of Proteins		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: E. A. Peterson Chief, Protein Chemistry Section LB NCI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH  Laboratory of Biochemistry		
SECTION  Protein Chemistry Section		
INSTITUTE AND LOCATION  NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  2.0	PROFESSIONAL:  1.0	OTHER:  1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Methods for the fractionation and analysis of proteins are developed and applied to the purification of specific proteins for the study of their function and structure. Displacement chromatography is being developed for the fractionation of macromolecules and particles of biological interest, employing <u>polyanions</u> differing in number of charges per molecule as displacers. Recent efforts have been directed toward the fractionation of <u>nonhistone proteins</u> of calf thymus nuclei and <u>marker proteins</u> in human serum. The procedure is particularly advantageous when large amounts of source material must be used to obtain sufficient amounts of a minor component, since the resolving power of the system can be focused on the narrow range of affinity represented by the protein of interest and its nearest neighbors. Recent tests have shown that the system is capable of separating genetic variants that differ in isoelectric point by 0.1 pH unit.		



## Project Description

### Objectives

To develop methods for the separation and analysis of proteins that will overcome present limitations and to apply these methods to the purification of specific proteins for the study of their functions.

### Methods Employed

Protein fractions separated by ion-exchange displacement chromatography were evaluated by gel electrophoresis, using both denaturing and nondenaturing conditions. Carboxymethyldextrans (CM-Ds) having a variety of degrees of substitution were prepared by reaction of alkaline dextran (nominally 10,000 M.W.) with chloroacetic acid.

### Major Findings

To test the resolving power of the narrow-range CM-Ds prepared by fractionating the heterogeneous products obtained from the original reaction, they were applied to the resolution of proteins known to have very similar isoelectric points.  $\beta$ -Lactoglobulins A and B are genetic variants that differ only in that A contains 56 carboxyl groups per molecular of 36,000 daltons and B has 54; their isoelectric points differ by about 0.1 pH unit. As a consequence they are difficult to separate by elution, though it has been done. They were easily separated by displacement chromatography on DEAE-cellulose, using a narrow-range CM-D, nearly all of which emerged in the effluent within and between the two peaks. This CM-D was selected on the basis of a preceding experiment employing five unfractionated CM-Ds that covered a wide affinity range but were effective in separating A and B, though not nearly so completely as the narrow range CM-D. Most of the CM-D in this case emerged in front of and behind the proteins. The fraction between the peaks gave a  $1/V_p$  of 11. (See the report for 1979-80 for a description of this measurement). Two unfractionated CM-D preparations with  $1/V_p$  values of 10.8 and 12.3, used in proportions that gave them a weighted average value that was the same as that of the narrow-range CM-D (11.8), were only somewhat more efficient than the quintet, indicating the widely overlapping ranges of affinity represented by the  $1/V_p$  values of unfractionated preparations.

Commercial ovalbumin claimed by the manufacturer to be 99% ovalbumin on the basis of electrophoretic analysis proved to comprise several more components than the three forms containing 0, 1, and 2 phosphate groups, respectively, that were initially expected. However, recent reports in the literature demonstrate heterogeneity based on differences in the carbohydrate content and this, as well as the presence of dimer, may account for the multiplicity observed. Similar profiles have recently been obtained by other investigators on an analytical scale in demonstrating the high resolving power of a new ion-exchange HPLC column, although the components were not characterized electrophoretically. In our work, the protein load was 50 times as high, relative to the size of the column.



Proteins can be separated from the CM-Ds that accompany them in the effluent fractions by lowering the pH to the vicinity of the protein's isoelectric point and passing the solution through a column of DEAE-cellulose equilibrated with  $\text{NaH}_2\text{PO}_4$ . The protein passes through unadsorbed, leaving the CM-D bound to the column. Preliminary experiments indicate that serial application of the fractions in this manner to the same column may be used to reclaim the spacer CM-Ds for re-use, while collecting the desired protein products. This promises to be of value in large scale separations, but at any scale this is a convenient way to obtain protein fractions free of CM-D if that is necessary for assay of an activity. This is an alternative to our previously described procedure wherein the protein is adsorbed to CM-cellulose and the CM-D passes through unadsorbed.

Preparation of narrow-range CM-Ds has been simplified and improved by preparing the full range of CM-D from one reaction mixture, removing portions continuously to produce a continuum of affinities. A linear rise in temperature was utilized to straighten out the otherwise convex reaction curve, so that equal intervals of time would encompass approximately equal increments in the incorporation of carboxymethyl groups. Since the amount of CM-D needed for a given spacing in displacement separations is much greater in the low-affinity region than in the later parts of the train, the reaction mixture was pumped out of the reactor very rapidly at the start of the reaction period and more slowly as the reaction proceeded. To simplify purification, the product was collected in only three or four fractions, and these (after purification from other reaction products) were fractionated by the method mentioned in the report for 1979-80 to obtain highly concentrated, salt-free, narrow-range CM-Ds.

Preparation of the full range of CM-Ds from a single reaction mixture not only provided a continuous array of CM-Ds but also eliminated a problem posed by the appearance of spurious (nonprotein) peaks of absorbance at 280nm in displacement chromatograms of simple protein mixtures when narrow-range CM-Ds were used that had been fractionated from preparations made individually with amounts of reagents selected to limit the incorporation of carboxymethyl groups to the desired levels. The offending components could be removed by treatment of such CM-D with decolorizing carbon before use, but the suppression of their formation is a more satisfactory solution. Evidently, high reagent concentrations in a time-limited reaction prevented sensitive portions of the dextran from being converted to structures that absorbed strongly at 280 nm.

It was necessary to reduce chloride in the initial CM-D preparations to very low levels in order to have the CM-Ds emerge in the fractionation displacement train at their natural concentrations. Chloride was more tightly bound than the lowest-affinity CM-Ds, and when it was displaced by later CM-Ds, a dip appeared in the CM-D profile because the chloride was taking the place of a more massive amount of CM-D. In the absence of chloride the concentration of the displaced CM-D was inversely proportional to its affinity as measured by  $1/V_p$  (See the report from 1979-1980). Chloride could be eliminated completely from the initial CM-D preparations by treatment with small amounts of coarse Dowex-1(OH), without detectable loss of CM-D.

Significance to Cancer Research (Objective 2, Approach 3)

Displacement chromatography of proteins promises to be of value at any scale of operation and therefore has significance to all research that involves the isolation of such substances. The high capacity, resolving power, and convenience of these systems offer to expedite the recognition and isolation of minor protein components such as regulating factors and marker proteins of interest in disease. The anticipated development of a systematic, general procedure for the purification of nonhistone nuclear proteins that participate in the regulation of the transcription of genetic information would be of substantial significance to cancer research since defective control of these processes appears to be involved in cancer.

Proposed Course of Research

Development of displacement systems for the separation of nonhistone nuclear proteins will be continued. A large scale isolation of HMG-1 and HMG-2 will be undertaken and the LMG proteins will be fractionated into several groups with selected narrow-range spacers.

Displacement chromatography will be applied to the purification of enzymes and other proteins of interest to members of this Laboratory. Preliminary trials on tissue cytosols have been very promising. Among these applications will be the isolation of serum and cytoplasmic factors mediating bone marrow response to inflammation in the guinea pig. Possible application in the fractionation of leukocyte granules and cell populations will be explored, using appropriate adsorbant matrices. Also the narrow-range CM-Ds will be tested as spacers in analytical separations on HPLC ion-exchange columns in the hope that they will improve resolution as well as eliminate the need for gradients. They will also be tested in thin layer chromatography of enzymes.

A system of CM-Ds will be prepared from 5000 MW dextran instead of the usual 10,000 MW material. Anticipated advantages, especially for the low-affinity range, are less heterogeneity in the initial preparations, less mass required for spacing, and a possible improvement in resolution.

Publications:

Torres, A.R., Krueger, G.G. and Peterson, E.A.: Two-dimensional gel electrophoresis can be used to examine chromatographic effluent fractions from displacement column chromatography. Clin. Chem. 28: 998-999, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 00945-09 LB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Factors Regulating the Synthesis of Collagen in Normal and Transformed Cells		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	B. Peterkofsky, Ph.D. Research Chemist	LB NCI
OTHER:	U. Chauhan, Ph.D. Visiting Fellow	LB NCI
	N. Blumenkrantz, Ph.D. Visiting Scientist	LB NCI
	G. Majmudar, Ph.D. Visiting Fellow	LB NCI
	J. M. Phang, Ph.D. Senior Investigator	MB NCI
	R. Spanheimer, M.D. Guest Worker	LB NCI
COOPERATING UNITS (if any)  None		
LAB/BRANCH  Laboratory of Biochemistry		
SECTION  Biosynthesis Section		
INSTITUTE AND LOCATION  DCBD, NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 5.25	PROFESSIONAL: 3.25	OTHER: 2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) We are studying the role of ascorbic acid and neoplastic transformation in regulating collagen metabolism in animal cells. Deficiency of ascorbate in guinea pigs leads to decreased proline hydroxylation and defective collagen synthesis, but in different time sequences. The mechanism by which synthesis is decreased is being investigated. The mechanism by which the proportion of type III collagen increases in Kirsten sarcoma virus transformed BALB 3T3 cells is also being determined.		

**Objectives:** To elucidate the basic mechanisms of collagen synthesis and secretion and to investigate the factors which regulate these processes in normal and transformed cells.

Methods Employed:

1. Proteins of whole chick embryos, isolated bones of these embryos, or cell-free systems derived from these bones or cultured fibroblasts are labeled with  $^{14}\text{C}$ -proline. The proteins are precipitated with trichloroacetic acid, redissolved and then assayed for radioactivity in collagen and noncollagenous proteins by a method involving specific digestion of collagen in a mixture of proteins using highly purified collagenase. This method was developed in our laboratory. The relative rate of collagen synthesis can be calculated from data obtained by this method and the specific rate of synthesis in tissues or cells is calculated by determining the DNA content and expressing the rate as cpm/ $\mu\text{g}$  DNA. The proteins of the cell and medium fractions of cultured fibroblasts are analyzed separately in order to study secretion. Morphological changes in cultured cells are recorded by polaroid photomicroscopy.

2. The level of proline hydroxylation in collagen of cells or tissues is measured by a new dual labeled proline method which was devised in this laboratory and eliminates the necessity for hydrolysis. To measure the level of lysine hydroxylation, cells or tissues are labeled with radioactive lysine, and hydrolyzed collagenase digests are chromatographed on Dowex-50 in  $2\text{N HCl}$ , which separates lysine and hydroxylysine.

3. Prolyl and lysyl hydroxylases are measured by  $^3\text{H}_2\text{O}$  release from  $^3\text{H}$ -proline or  $^3\text{H}$ -lysine labeled unhydroxylated collagen prepared by incubating chick embryo frontal bones with the labeled amino acid in the presence of the iron chelator  $\alpha,\alpha$ -dipyridyl, which inhibits hydroxylation.

4. Collagen types synthesized in cultured cells are determined by analysis of  $^{14}\text{C}$ -or  $^3\text{H}$ -proline labeled, denatured collagen using electrophoresis in sodium dodecyl sulfate-polyacrylamide slab gels. Disulfide bonds are detected by running samples with and without dithiothreitol and observing alterations in the positions of  $\alpha$  chains. Radioactive proteins are detected by fluorography.

Cell lines used in these studies:

Chick embryo fibroblasts: prepared by digesting frontal bones from 15 day chick embryos with crude collagenase and culturing the released cells. Subcultures in the second to fifth passage are used.

L-929--a line of mouse embryo fibroblasts established about 30 years ago by use of a chemical carcinogen.

BALB-3T3--a contact inhibited line of mouse embryo fibroblasts isolated by Todaro and Aaronson and subclones of this line, P3 and 714, which are more stringently contact-inhibited than the original culture, and P13, which has lost density dependence.



Ki-3T3-234---a line established by Aaronson by transformation of BALB 3T3 cells with Kirsten sarcoma virus. These cells do not produce viral particles unless super-infected with murine leukemia virus.

Mo-3T3--BALB 3T3 cells transformed with Moloney sarcoma virus.

SV-3T3--BALB 3T3 cells transformed with SV-40 virus.

NQT-3T3-714--BALB 3T3 cells transformed with a chemical carcinogen, 4-nitroquinoline-1-oxide.

Embryonic and adult human diploid fibroblasts are purchased commercially.

BALB 3T3 subclones 3 and 714 were transformed with a temperature sensitive mutant of Kirsten sarcoma virus (KSV) containing Moloney leukemia virus (MLV) helper to give a mass infected and transformed line, tsKi(MLV)-3T3. Control lines infected with only MLV were also produced (MLV-3T3). A nonproducer subclone, tsKi-3T3-714 was derived from the transformed culture of 3T3-714.

### Major Findings:

#### I. The role of ascorbic acid in collagen metabolism.

- A. Determination of the mechanism by which the percentage of net production of collagen is decreased in calvarial bone from scorbutic guinea pigs.

Net production is the result of concomitant biosynthesis and degradation. We had found that net production of collagen relative to net production of total protein in cultures of calvarial bone from scorbutic guinea pigs was decreased to about 40% of controls. This could have resulted from either a decrease in the absolute rate of production of collagen or an increase in the absolute rate of production of noncollagenous proteins. Absolute rates were determined by measuring the specific radioactivity of the  $^{14}\text{C}$ -proline precursor in the amino acid pool of bone, and it was found that net production of collagen was specifically decreased. Decreased production was not due to increased collagen degradation but rather to decreased synthesis. This was determined by measuring low molecular weight hydroxyproline, which is specifically produced during collagen degradation. The absolute rate of degradation was calculated using free proline specific radioactivity, and it was found not to differ significantly in control and scorbutic bone.

Several observations suggested that the effect of ascorbate deficiency on proline hydroxylation and collagen synthesis might be unrelated, contrary to our previous proposals:

1. Proline hydroxylation was decreased maximally (from 45% down to 30%) in bone from scorbutic guinea pigs which had been on the experimental diet for two weeks, but at that time collagen synthesis was unaffected; collagen synthesis did not decrease significantly until after the second week and was at the lowest point (40% of control) by the fourth week.



2. Decreased proline hydroxylation, but not decreased collagen synthesis, could be returned to normal by treatment of bone from scorbutic animals with ascorbate in vitro.
3. Addition of  $\alpha, \alpha'$ -dipyridyl inhibited proline hydroxylation in bone from normal animals without decreasing collagen synthesis.

Therefore, we investigated other factors which might be affecting collagen synthesis. Ascorbate deprivation leads to decreased food intake and weight loss almost precisely after two weeks, which is probably the cause of death of scorbutic animals during the fifth week. We considered the possibility that these effects might be related to the observed decrease in collagen synthesis in scorbutic animals. Collagen synthesis was measured in bone from scorbutic guinea pigs which were either rapidly losing weight or had merely stopped gaining weight during the third week on the scorbutogenic diet. Comparison was made to a food-restricted control which was fasted for alternating 24 hr periods to prevent weight gain, and to an ad libitum-fed control animal, both of which received a 50 mg ascorbate supplement. The ad libitum-fed control had the highest relative rate of bone collagen synthesis (20%) while the rate was lowest (6%) in bone from the scorbutic animal rapidly losing weight. Rates in bone from a scorbutic animal which was neither losing nor gaining weight and the food-restricted control were similar (13-15%) and significantly lower than the ad libitum-fed control. These results suggest that a specific decrease in collagen synthesis results when food intake is decreased and weight gain prevented. Such an effect may be induced by ascorbate deprivation and explain the often observed defects in connective tissue of scorbutic animals.

#### B. Formation of proline metabolites in bone

In the course of establishing the methodology to measure collagen degradation, we found that metabolites were formed from radioactive proline in chick embryo frontal bone. They interfered with measurement of radioactive hydroxyproline by a commonly used method of ion-exchange chromatography on Dowex-50 columns. The major metabolite was identified as glutamic acid by its chromatographic and crystallization properties. It was eluted from AG50 cation exchange resin with 1.0 N HCl in the hydroxyproline region, but was separated from hydroxyproline on a DC-6A column in the amino acid analyzer. Another metabolite was identified as aspartic acid. It was not separated from hydroxyproline on either AG50 using 1 N HCl for elution or on DC-6A using 0.1 M sodium citrate, pH 3.25 for elution, but adequate separation was obtained by elution with 0.2 M sodium citrate buffer at pH 2.91. Formation of these metabolites was not related either to protein synthesis or proline hydroxylation. Therefore, it is possible to analyze for hydroxyproline accurately by using a separate unhydroxylated sample to correct for the presence of metabolites. The formation of glutamic acid suggested that proline oxidase activity might be present in bone tissue, but none was detected using a sensitive radioisotopic assay.

Although the amount of radioactivity found in the metabolites was 36% of the amount of [ $^{14}\text{C}$ ]proline incorporated into protein, no radioactive glutamic or aspartic acid was present in protein hydrolyzates. This observation suggests that the metabolites did not enter the major amino acid pool used for protein synthesis.

## II. Modification of collagen phenotype by transformation.

- A. We examined the possibility that retention of the high proportion of type III, relative to type I, procollagen, in a temperature-sensitive Kirsten sarcoma virus transformant grown at a nonpermissive temperature, might be due to the fact that mRNA for type III was long-lived. tsKi-3T3 cells were treated for varying time intervals with Actinomycin D to inhibit RNA synthesis, pulse-labeled with  $^{14}\text{C}$ -proline for 1 hr and the relative rate of procollagen synthesis determined. Incorporation into procollagen decreased with time of exposure to the inhibitor, suggesting a half-life for total procollagen mRNA of 4 hr. The half-life was similar at permissive and nonpermissive temperatures. In addition, the procollagen mRNA half-life was almost identical in the parent 3T3-714 cells, which synthesize type I procollagen almost exclusively. This suggests that the half-life of type III mRNA does not differ significantly from that of type I and, therefore, that retention of type III procollagen synthesis at the nonpermissive temperature cannot be explained by differing half-lives.

- B. Aerobic glycolysis and glucose transport in transformed cells.

During the course of studying collagen metabolism in various transformed BALB 3T3 cell lines, we observed that unlike some virally transformed lines we carried, a nitroquinoline oxide transformant, NQT-3T3-714, did not acidify the culture medium even at high cell densities. In view of the fact that the concept of tumor cells exhibiting high rates of aerobic glycolysis (lactate formation) is still widely accepted, we explored this observation further. We measured lactate production in a series of BALB 3T3 subclones and transformants derived from them and also investigated how glycolysis and the rates of glucose transport were related in these cells. NQT-3T3-714 and a temperature-sensitive Kirsten sarcoma virus (tsKi-3T3-714) transformant of subclone 714 exhibited transformed phenotypes with respect to morphology and growth properties, but their rates of lactate production and [ $^3\text{H}$ ]deoxyglucose uptake were similar to those of the parent cells. Inhibitors of oxidative phosphorylation increased glycolysis several-fold in these transformants, showing that there was no defect in the enzymes of this pathway. At a temperature nonpermissive for transformation of ts-Ki-3T3-714, lactate production by this line did not decrease relative to the rate of the parent cells. Another transformant, Ki-3T3-234, had a glycolytic rate which was four-five times greater than the low lactate producers while other transformants exhibited intermediate rates, and the rate of a third nontransformed 3T3 A31 subclone, K-1-1, was comparable to the rate of Ki-3T3-234. The rates of [ $^3\text{H}$ ]deoxyglucose uptake by this series of cells were closely proportional to their glycolytic rates rather than to their state of transformation. Increasing glycolysis by oligomycin or

dinitrophenol, however, did not cause a concomitant increase in sugar uptake. Neither glycolysis nor deoxyglucose uptake in the high lactate producer (Ki-3T3-234) was inhibited by ouabain, suggesting that  $\text{Na}^+\text{K}^+/\text{ATPase}$  is not a regulator of these functions in 3T3 cells. In 3T3-derived cells, it appears that the rates of glycolysis and glucose uptake may be regulated in tandem under some conditions, and that neither process is an obligatory consequence of neoplastic transformation.

#### Proposed Course of Research:

##### I. Role of ascorbate in collagen metabolism.

###### A. Effect of fasting on collagen production in normal animals supplemented with ascorbate.

Since the decrease in collagen synthesis in bone of scorbutic animals may be related to the decrease resulting from food restriction, we will examine the mechanism of the latter phenomenon. This "fasting" effect in itself may be an important regulatory mechanism in animals. The effect will be characterized by determining the interval of fasting required to inhibit collagen production, the influence of sex, age, and other factors, and whether the effect is specific for bone tissue. The molecular mechanism will be examined by extracting RNA from bone of control and fasted guinea pigs and measuring translatable procollagen mRNA levels in a reticulocyte lysate system. If the level of translatable mRNA is decreased in proportion to the effect on collagen synthesis, this would indicate that a collagen degradative mechanism is not involved. This result also would lend support to the theory that the fasting effect is related to the effect of ascorbate deficiency on collagen synthesis.

###### B. Influence of decreased food intake and weight loss on collagen synthesis in scorbutic animals

The major objective in this approach will be to eliminate the weight loss observed after two weeks on an acutely scorbutogenic diet. By eliminating this factor, we should be able to determine whether the decreased collagen synthesis caused by ascorbate deficiency was due to the "fasting" effect or to a long-term effect of decreased proline hydroxylation. Other models which might eliminate weight loss while retaining the inhibitory effect on proline hydroxylation will be examined. These would include chronic scurvy, achieved by supplementing animals with low doses of ascorbate, and *in vivo* reversal studies in which ascorbate is refed after inducing acute scurvy for four weeks. The molecular mechanism for decreased collagen synthesis will be investigated as described for the "fasting" effect.

##### II. Alteration of collagen phenotype

Experiments to determine the half-life of types I and III procollagen mRNA will be continued. The procollagens produced in pulse-labeled control and Actinomycin D-treated tsKi-3T3-714 cells grown at permissive and nonpermissive temperatures will be examined on SDS-PAGE and the proportions of

labeled  $\alpha$ -chains determined. In addition, RNA will be extracted from such cells and the concentration of translatable mRNA for types I and III procollagen chains measured by examining the translation products on gels or by immunoprecipitation.

#### Significance to Biomedical Research and the Program of the Institute:

Determining the precise mechanism by which vitamin C affects collagen synthesis should lead to a more accurate use of this vitamin. In addition, our studies with transformed cells indicate that, at least under some conditions, animal cells may be able to synthesize a substitute for the function of vitamin C in connective tissue.

The alteration of the collagen phenotype by transformation provides further information on biological changes caused by the carcinogenic agents as well as useful models for studying cellular differentiation.

#### Publications:

Peterkofsky, B.: Bacterial collagenase. In Methods in Enzymology, Vol. 82, Cunningham, L.W. and Fredriksen, D.W. (ed.), Academic Press, N.Y. pp. 453-471, 1982.

Bateman, J. and Peterkofsky, B.: Mechanisms of Kirsten murine sarcoma virus transformation—induced changes in the collagen phenotype and synthetic rate of BALB 3T3 cells. Proc. Natl. Acad. Sci. USA 78: 6028-6032, 1981.

Chojkier, M., Bateman, J., Phang, J.M., and Peterkofsky, B.: Formation of proline metabolites in chick embryo bone: interference with the measurement of free hydroxyproline by ion-exchange chromatography. Anal. Biochem. 120: 330-338, 1982.

Peterkofsky, B. and Prather, W.: Correlation between the rates of aerobic glycolysis and glucose transport, unrelated to neoplastic transformation, in a series of BALB 3T3-derived cell lines. Cancer Res. 42: 1809-1816, 1982.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05202-15 LB
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Isolation, Fractionation, and Characterization of Native Nucleoproteins</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	O. Wesley McBride	Medical Director      LB NCI
OTHER:	Usha Kasid Adina Breiman	Fogarty Fellow      LB NCI Fogarty Fellow      LB NCI
COOPERATING UNITS (if any) <p style="text-align: center;">Dr. Philip Leder, LMG, CH Drs. David Swan &amp; Stuart Aaronson, LCMB, NCI</p>		
LAB/BRANCH <p style="text-align: center;">Laboratory of Biochemistry</p>		
SECTION <p style="text-align: center;">Protein Chemistry Section</p>		
INSTITUTE AND LOCATION <p style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	2.7	2.8
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <p>             The purpose of this project is to develop methods for <u>gene transfer</u> to mam-              malian cells and to use these techniques for <u>gene mapping</u>, analysis of <u>gene</u>  <u>expression</u>, and <u>cloning eukaryotic genes</u>. A large number of independent  <u>somatic cell hybrid lines</u> segregating human chromosomes have been isolated and              the human chromosome content of each line determined. Analysis of these lines              with isotopically labeled <u>cloned DNA probes</u> has allowed assignment to specific              human chromosomes of the human cellular analogues of four viral <u>onc genes</u> as              well as <u>kappa</u>, <u>lambda</u> and heavy chain <u>immunoglobulin (Ig)</u> genes and four Ig  <u>pseudogenes</u>. Heavy chain V, D, J and C genes have all been <u>regionally localized</u>              to a single chromosomal band (14q32) and one onc gene has been regionally              assigned on chromosome 6. <u>Chromosomal assignment</u> of these genes now permits              a rational assessment of the possible role of onc genes in human cancer.  <u>DNA-mediated gene transfer</u> is being used in combination with <u>recombinant DNA</u>  <u>techniques</u> to isolate and analyze the human <u>tk gene</u>. Transformants resulting              from <u>chromosome-mediated gene transfer</u> have been characterized and used to map              repetitive DNA sequences on the human X chromosome.           </p>		



## Objectives

Development of methods for gene transfer to mammalian cells and use of these procedures for chromosomal and regional gene mapping, analysis of gene expression in normal and neoplastic cells and cloning eukaryotic genes.

## Methods Employed

Tissue culture procedures, including plating in selective media, cloning, selection of cell mutants, and isolation of somatic cell hybrids after inter-specific fusion of microcells with whole cells or cell/cell fusion. Metaphase chromosome isolation and purification by isopycnic and velocity sedimentation; gene transfer to mammalian cells by uptake of purified metaphase chromosomes or high molecular weight, eukaryotic DNA with subsequent isolation of biologically transformed colonies in selective media. Isolation and characterization of gene products by chromatography, electrophoresis, isoelectric focusing, and immunochemical procedures. Karyological analysis of hybrid cells and transformed cells by fluorescence, phase, and bright-field photomicroscopic techniques. Isolation of DNA and RNA, analysis of DNA reassociation kinetics, preparation of recombinant DNA, and cloning recombinant DNA in prokaryotes.

## Major Findings

**I. Somatic Cell Hybridization.** Analysis of somatic cell hybrids segregating human chromosomes permits the localization of human genes to specific chromosomes. Several large series of independent hybrid cell lines were isolated in selective medium containing HAT (hypoxanthine, aminopterin, and thymidine) and 100  $\mu$ M ouabain after polyethylene glycol 1000 induced fusion of human cells with hprt<sup>-</sup> and tk<sup>-</sup> mutant rodent fibroblasts. The human parental cells included Hela, normal WI38 cells, and well characterized fibroblast lines (from Camden Institute for Medical Research) containing normal karyotypes except for the presence of specific reciprocal translocations [t(2; 6) = GM2658; t(X; 14) = GM0073; t(17; 22) = GM119 and GM3196; t(13; 17) = GM1663] or deletions [del(13) (pter > q14 :: q22 > qter) = AG1142]. Hybrid lines were also isolated after fusion of rodent fibroblasts with peripheral leukocytes from a patient with Chronic Myelogenous Leukemia (CML), and our karyotyping demonstrated that these donor cells contained the Philadelphia chromosome [t(22; 9)]. The human parental donor lines were specifically chosen for the presence of appropriate translocations or deletions which permit chromosomal regional localization of immunoglobulin genes and/or cellular oncogenes, and to provide resource for the future molecular cloning of DNA segments adjacent to these genes. The other criteria for selection of human donor lines were the presence of translocations permitting both selective retention and loss of appropriate chromosomal translocation fragments and the ability to construct hybrids in which both reciprocal translocation products and the corresponding non-translocated homologues could be identified by isoenzyme analysis. Cloned hybrid lines were subcloned once or more to obtain segregant hybrid cell populations containing a reduced, and relatively homogeneous, content of specific human chromosomes. The specific human chromosomes present in hybrid cell lines and subclones were determined by isoenzyme analyses and sometimes confirmed by karyotyping. In collaboration with Dr. David Swan, DNA was simultaneously isolated from these same hybrid cell populations, and DNA fragments were trans-

ferred to nitrocellulose after restriction endonuclease digestions and agarose gel electrophoresis. Hybridization of the transferred DNA with isotopically labeled, cloned DNA probes thereby permitted assignments of genes to specific human chromosomes and chromosome segments.

A. Chromosomal localization of human immunoglobulin genes and pseudogenes. In collaboration with Dr. Philip Leder and his colleagues, several immunoglobulin genes and pseudogenes have been assigned to specific human chromosomes. Probes were isolated and nucleic acid blot hybridizations were performed by Dr. Leder et al. The kappa and lambda constant region Ig genes were assigned to human chromosomes 2 and 22, respectively. Probes for Ig heavy chain genes were used to regionally map these loci in somatic cell hybrids containing the X; 14 translocation. Heavy chain variable region ( $V_H$ ), diversity region ( $D_H$ ), joining segments ( $J_H$ ) and epsilon constant region ( $C_\epsilon$ ) genes were all localized to a single band (14q32) at the telomere of human chromosome 14. This band represents less than 10,000 Kb and it can be estimated that the heavy chain Ig genes occupy at least 1000 Kb of this region. Hence, these genes occupy a substantial fraction of this entire band but we have identified at least one non-Ig gene, brain type creatine kinase (CKBB), on this same chromosome segment. Variable region kappa genes have been regionally localized to the centromeric region of chromosome 2p by in situ hybridization in another laboratory. Regional mapping of  $C_K$  and  $V_K$  is in progress using the 2; 6 translocation hybrids to confirm the regional assignment. A lambda pseudogene ( $\lambda\psi 1$ ) and a 9Kb BamHI epsilon pseudogene ( $\psi\epsilon 2$ ) have been assigned to human chromosomes 18 and 9, respectively. Both these pseudogenes bear evidence of a RNA-type intermediate in their formation, including clean loss of intervening sequences, coincident homology precisely to the site of poly(A) addition, and poly(A) tails. They differ from processed mRNAs in lacking the V region, and both contain short direct terminal repeats similar to transposable elements in prokaryotes and eukaryotes. Another epsilon pseudogene ( $\psi\epsilon 1$ ) maps to chromosome 14q32 as does the epsilon gene. A pseudogene of the diversity region has been assigned to chromosome 15.

B. Chromosomal mapping of human onc gene analogues. Acute transforming retroviruses arise by recombination of replication competent type C RNA viruses with evolutionarily well conserved cellular genes termed onc genes. A large body of recent circumstantial evidence strongly suggests that activation of any of these cellular onc genes may lead to neoplastic transformation. Knowledge of the chromosomal location of these onc genes could greatly facilitate assessment of the possible role of these sequences in the etiology of human cancer. Dr. Stuart Aaronson and colleagues have molecularly cloned the human analogues of these viral onc genes. They prepared isotopically labeled probes from these cloned genes for use in Southern blotting analysis of DNA from our somatic cell hybrid lines. This method has been used to assign the human onc gene analogue (c-sis) of the only known transforming retrovirus of primate origin (simian sarcoma virus, SSV) to human chromosome 22. The human analogue of the Moloney murine sarcoma virus transforming gene (v-mos) and the avian myeloblastosis virus onc gene (v-myb) were assigned to chromosomes 8 and 6, respectively. We have regionally assigned c-myb to the long arm of chromosome 6 distal to band q15. A transforming gene from a human bladder carcinoma (blc)

has been cloned by Aaronson et al. and we have mapped it to chromosome 11. This gene has homology with the transforming gene (bas) from the BalbC murine sarcoma virus. Chromosomal mapping of several other onc genes has not been completed.

C. Regional chromosomal mapping of other genes. Regional localization of creatine kinase (CKBB) to 14q32 and of the gene for soluble malic enzyme (ME-1) to 6q13-q15 has been obtained from our studies.

II. Molecular Cloning of Human tk Gene. Progress in cloning the tk gene has been somewhat limited due to changes in laboratory personnel. The primary approach has involved serial transfer of human DNA to tk<sup>-</sup> mouse cells and isolation of mouse transformants containing rare human DNA sequences including the human tk gene. Transformant DNA was isolated, sheared, size fractionated, tailed with dG-residues and annealed to (dC)-tailed Charon 4 arms. The recombinant DNA was incubated with  $\lambda$  phage packaging extract and used to transfect *E. coli*. The resulting small library was screened by colony hybridization with a [<sup>32</sup>P]-labeled repetitive human DNA probe. No recombinants containing human inserts were detected in this small library. Further control experiments were performed to maximize the sensitivity for detection of recombinants containing human inserts and to minimize false positive plaques. Size fractionated 15-20 KB fragments have been isolated from a partial Mbo digest of transformant DNA, ligated with purified BamHI Charon 30 arms, packaged, and used to transfect *E. coli*. A library of at least 500,000 recombinants is undergoing screening by colony hybridization to detect recombinants containing human inserts and the human tk gene.

III. Analysis of Chromosome-Mediated Gene Transfer (CMGT) Transformants. We have previously shown that functional fragments of donor chromosomes are transferred to recipient cells by CMGT. The amount of transferred DNA was quantified by nucleic acid hybridization analysis in a group of 12 transformants selected for hprt transfer. The transferred DNA ranged from less than 2% to 20% of the human X chromosome (i.e. less than 0.1% to 1% of the haploid genome). These extensively characterized transformants are now valuable for other types of analysis. We have recently started a collaboration with Dr. David Housman at MIT to analyze these transformants for the number and distribution of a class of dispersed repetitive human DNA sequences which are present in a copy number of about 1000 per haploid genome. After digestion of human X chromosomal DNA with restriction endonucleases which do not cleave within the repetitive sequences and Southern blotting, a large series of hybridizing bands are observed. Examination of four transformants indicated that the number of bands is roughly proportional to the human X chromosome DNA content. One band was present in all transformants and presumably it was the sequence in this repetitive family which is located closest to the hprt gene. Additional transformants are being analyzed and they will be used to map the distribution of these repetitive sequences on the X chromosome with particular emphasis on the telomeric portion between the hprt and g6pd genes.



Proposed Course of Research

Chromosomal assignment and regional localization of the human cellular onc gene analogues, immunoglobulin genes, and Ig pseudogenes will be completed. These same hybrid cell lines will also be used to map other genes of interest. It will be determined whether onc gene analogues are localized on the chromosomal segments involved in very specific translocations in certain human cancers including CML and Burkitt's lymphoma. Most importantly, appropriate neoplastic cells will be analyzed for the level of transcription of these onc genes.

The human tk gene will be cloned by the "reiterated human DNA probe approach". A transformant line containing the distal segment of the human X chromosome including the hprt and g6pd genes will be provided to Dr. David Housman for his efforts to clone the human g6pd gene by this same approach. In addition, in this laboratory we will attempt to purify the human chromosome fragment from this hprt<sup>+</sup>, g6pd<sup>+</sup> cell line by velocity sedimentation and fluorescence activated chromosome sorting prior to DNA isolation and cloning in both phage and cosmid vectors.

The Philadelphia (Ph<sup>+</sup>) chromosome will be isolated from metaphase chromosome preparations of CML peripheral leukocytes by velocity sedimentation and fluorescence activated chromosome sorting. An intact human chromosome 22 will be isolated by similar procedures from a human/hamster hybrid cell line containing only this single human chromosome. Recombinant DNA libraries will be constructed with DNA from the Ph<sup>+</sup> chromosome and chr. 22 in phage and cosmid vectors. All DNA sequences from the Ph chromosome can be obtained in a cosmid library containing about 1000 recombinants. Restriction mapping of these libraries will be started with the major emphasis on determining and analyzing sequences at the Ph chromosomal break point.

Significance to Cancer Research (Objective 2, Approach 3)

The regional localization of the heavy chain Ig genes to the break point (14q32) of the reciprocal 8/14 translocation reported in many Burkitt's lymphomas supports the hypothesis that B cell neoplasms may arise through activation of an onc gene on chromosome 8 by a translocated active Ig gene. The other two reciprocal translocations found in B cell neoplasms are 2/8 and 8/22 and involve chromosomes bearing kappa and lambda genes, respectively, although regional localization of the light chain genes has not been obtained yet. Assignment of onc gene analogues to specific human chromosomes provides a basis for testing the hypothesis that activation of these genes may have a role in human cancer. This requires measurement of the level of transcription of the appropriate onc gene in neoplastic cells containing specific translocations of the onc bearing chromosome.

Publications

1. McBride, O.W., Olen, A.S., Aulakh, G.S. and Athwal, R.S.: Measurement of transcribed human X-chromosomal DNA sequences transferred to rodent cells by chromosome-mediated gene transfer. Mol. Cell. Biol. 2: 52-65, 1982.
2. McBride, O.W.: Techniques of chromosome-mediated gene transfer. In: Techniques in Somatic Cell Genetics, J.W. Shay, Ed., Plenum Press, in press, 1982.
3. McBride, O.W., Heiter, P.A., Hollis, G.F., Swan, D., Otey, M.C. and Leder, P.: Chromosomal location of human kappa and lambda immunoglobulin light chain constant region genes. J. Exp. Med. 155: 1480-1490, 1982.
4. Hollis, G.F., Hieter, P.A., McBride, O.W., Swan, D. and Leder, P.: Processed genes: a dispersed human immunoglobulin gene bearing evidence of RNA-type processing. Nature 296: 321-325, 1982.
5. Battey, J., Max, E.E., McBride, O.W., Swan, D. and Leder, P.: A processed human immunoglobulin epsilon gene has moved to chromosome 9. Proc. Natl. Acad. Sci., in press, 1982.
6. Swan, D.C., McBride, O.W., Robbins, K.C., Keithley, D.A., Reddy, E.P. and Aaronson, S.A.: Chromosomal mapping of the simian sarcoma virus onc gene analogue in human cells. Proc. Natl. Acad. Sci. USA., in press, 1982.
7. Prakash, K., McBride, O.W., Swan, D.C., Devare, S.G., Tronick, S.R. and Aaronson, S.A.: Molecular cloning and chromosomal mapping of a human locus related to the Moloney murine sarcoma virus transforming gene. Proc. Natl. Acad. Sci. USA, in press, 1982.
8. McBride, O.W., Swan, D.C., Robbins, K.C., Prakash, K. and Aaronson, S.A.: Chromosomal mapping of tumor virus transforming gene analogues in human cells. Plenum Press, in press, 1982.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05203-14 LB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Immunochemical Purification and Characterization of Immunocytes and Components		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	M.G. Mage	Immunochemist LB NCI
OTHERS:	V. Alvarez	Expert LB NCI
	L.L. McHugh	Biologist LB NCI
COOPERATING UNITS (if any)  Laboratory of Microbial Immunity, NIAID		
LAB/BRANCH Laboratory of Biochemistry		
SECTION Protein Chemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Our goal is the development of <u>cell separation methods</u> for the specific isolation of immune cells, particularly for varieties of <u>antigen-reactive cells</u> (ARC) involved in cellular immune reactions, and for their subcellular fractionation in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. Populations of cells containing ARC are tested for binding to the cell surface antigens of target cells attached to insoluble supports. Separated populations are tested for <u>cytotoxic effector cells</u> (CTL) and their precursors, for activity in <u>allo-graft rejection</u> and <u>graft-versus host reaction</u> and in the <u>mixed lymphocyte reaction</u> . T cell subpopulations from thymus and spleen are also separated by and characterized with specific reagents such as peanut agglutinin and antibodies to the Lyl and CTL differentiation antigens. Surface molecules of target cells are isolated to test their binding to ARC.		

## Objectives

The goal of this project is the development of methods for the specific isolation of immune cells, particularly for different types of antigenreactive cells (ARC) involved in cellular immune reactions to alloantigens, to study the ontogeny of these cells, to study their roles, alone and in combination, in several in vitro and in vivo cellular immune reactions, and for their immunochemical characterization in order to study the mechanisms involved in the development of immune reactivities and immune macromolecules. In particular, we study the differentiation of cytotoxic effector cells (CTL) from precursors (CTLp) in thymus, the molecular reactions between immune cells and antigens bound to target cell surfaces, and the role of T cell subpopulations in in vivo cellular immune phenomena such as graft rejection and the graft-versus host reaction.

## Methods Employed

ARC, obtained from normal mice or from mice immunized with allogeneic tumor cells or normal cells are incubated on surfaces coated with allogeneic or syngeneic target cells or on surfaces coated with antibodies to cell surface molecules. Graft-versus host activity (GVH) of nonadherent cells or of released adherent cells is measured by the Simonsen spleen weight assay in neonatal F1 mice. CTL activity is measured by  $^{51}\text{Cr}$  release from target cells. Stimulation by alloantigen is measured by the mixed lymphocyte reaction and by generation of CTL in vitro.

T cell subpopulation from thymus and spleen are separated and characterized by their reactions with specific reagents such as peanut agglutinin and antibodies to the LyT and CTL (Rothstein et al. *J. Immunol.* 120: 209, 1978) differentiation antigens. Cell surface molecules of target cells are isolated to test their binding to ARC.

## Major Findings

### (1) Help for CTL generation is not antigen-specific.

Our previous development of a non-lytic separation technique that produces highly purified populations of  $\text{Lyt}2^+$  and  $\text{Lyt}2^-$  T cells allowed us to examine the antigen specificity of help for CTL generation. For this purpose,  $\text{Lyt}2^-$  T cells from BALB/c, C57BL/6, or B6CF1 spleens were added to BALB/c or C57BL/6 or B6CF1  $\text{Lyt}2^-$  responder cells in MLC, with semi-allogeneic B6CF1 B cells as stimulators. The BALB/c  $\text{Lyt}2^-$  cells, whether added to BALB/c or to C57BL/6  $\text{Lyt}2^+$  responder cells, had more helper activity than had C57BL/6 or B6CF1  $\text{Lyt}2^-$  cells, indicating a lack of antigen specificity for the help provided, a finding compatible with their postulated role of providing the lymphokine IL2.

### (2) Help is limiting in MLC of unfractionated Ig- responder cells.

Even when  $\text{Lyt}2^-$  helper cells are present in the MLC, additional CTL activity was generated when IL2 was added to the cultures, indicating that with the relative numbers of  $\text{Lyt}2^-$  and  $\text{Lyt}2^+$  cells present in unimmunized mice, the amount of CTL activity that can be generated is not limited by the numbers of CTL precursors, but rather by the amount of help that is provided by the  $\text{Lyt}2^-$  helper cells.

- (3) Lyt2<sup>-</sup> helper cells require the presence of plastic-adherent accessory cells in order to provide help for CTL generation.

We looked to see if the help provided by purified Lyt2<sup>-</sup> cells was dependent on the presence of accessory cells, because such dependence has been reported for IL2 production by such cells. When stimulator B6CF1 B cells and/or BALB/c Lyt2<sup>+</sup> responder cells were depleted of adherent cells by preincubation (prior to cell separation procedures) for ninety minutes at 37° in uncoated tissue culture dishes, little or no CTL activity was generated, even when Lyt2<sup>-</sup> responder cells were added. However, addition of IL2 restored the ability of such MLC to generate CTL activity, indicating that the help provided by Lyt2<sup>-</sup> cells is indeed dependent on the presence of adherent cells, and can be bypassed by the addition of their putative product, the lymphokine IL2.

#### Significance to Cancer Research (Objective 2, Approach 1)

The T cells that undergo maturation in the thymus are thought to be directly involved in anti-tumor immunity, by means of cell-mediated tumor rejection. Cytotoxic cell precursors, and helper and suppressor T cells are thought to undergo maturation in the thymus. Knowledge of the antigen-binding characteristics, differentiation antigens, and reactivities of immune cells at different stages of differentiation may help in developing specific purifications of various types of immune lymphocytes reactive against tumor antigens and in specific suppression of the graft versus host reaction, which is a serious iatrogenic complication of cancer therapy with bone marrow transplantation.

#### Proposed Course of Research

We plan to (1) continue to develop the general separation methodology based on specific binding of Ig-coated cells to anti-Ig-coated surfaces, (2) develop further separations of T cell subtypes, particularly CTLs, their precursors, helpers, and suppressors, and study their differentiation, (3) continue to study the contributions of these cells in vivo to immune reactions such as graft rejection, GVH reaction, and tumor rejection, (4) study the macromolecules involved in the binding of CTL to target cells, and (5) study the molecular requirements for immunogenic antigen presentation to CTLp.

Publications: None



## Project Description

### Objectives

In general, our objective is to understand the mechanism(s) of action of steroid hormones: how they act to kill some cells and not others, and how they induce specific proteins. When possible, we aim to apply our findings to clinical situations.

Specific sub-objectives are as follows:

1. Objective one is to study the mechanisms by which leukemic cells develop resistance to lysis by glucocorticoids and then to utilize resistant cells to define the steps of steroid action. For this purpose we use for the most part, the human CEM cell line, derived from a patient with acute lymphoblastic leukemia.
2. Objective two is to explore the mechanisms by which steroids (and other hormones) control the production of growth hormone and prolactin. For these studies we employ the rat pituitary adenoma cell line GH<sub>3</sub>. We know that hybrids between GH<sub>3</sub> and mouse L cells fail to make either peptide, and we are trying to define the nature of this control.
3. Objective three is to develop a technique for mass screening of cloned cDNA libraries for changes in specific cDNAs after inductive events.
4. Objective four is to purify human glucocorticoid receptors and produce antisera to them.
5. Objective five is to continue tests of deacylcortivazol as an antitumor cell agent.
6. Objective six is to develop in vitro transcription systems for growth hormone and prolactin genes.
7. Objective seven is to develop vectors containing all or parts of the growth hormone and/or prolactin genes and then to use these vectors to study the control of expression of these genes.
8. Objective eight is to study the glucocorticoid receptors of "New World" vs "Old World" monkeys.
9. Objective nine is to define the relationship between receptor occupancy and specific enzyme induction by glucocorticoids.

### Methods

In addition to the methods outlined in these reports earlier, we have added: high performance liquid chromatography, cell-free DNA transcription, and microinjection of single cells.



Major Findings

1. Objective one: We have compared the time course of steroid inhibition of growth in a series of wild-type clones obtained in the absence of selective pressures, directly from the uncloned CEM line. We observe that these clones vary widely in the time it takes for them to cease growing in the presence of steroid. Some take as long as four days. This fact is of great potential significance for clinicians attempting to use glucocorticoids to cure leukemia. We have also explored in some detail a single steroid-resistant CEM clone, CEM-C1, found among the randomly isolated clones. We have shown that CEM-C1 has chromosomal, cellular, and glucocorticoid receptor properties virtually identical to those of its steroid-sensitive sister clone CEM-C7. Yet CEM-C1 resists cell kill. We have employed the induction of glutamine synthetase as a marker for glucocorticoid receptor function. This enzyme is induced by active glucocorticoids in wild-type CEM cells and not induced in receptor-deficient mutants. Glutamine synthetase is induced in CEM-C1, indicating that these cells have intact receptors and placing the lesion in them which prevents lysis "beyond the receptor". They therefore represent a cell line of great potential value in defining steroid action.
2. Objective two: We have studied the content and anatomy of growth hormone genes from both rat (inducible) and mouse (noninducible) in rat x mouse (noninducible) hybrid cells. Restriction endonuclease hydrolysis was followed by Southern blotting of the electrophoresed products and hybridization with P<sup>32</sup> labeled GH cDNA. The rat gene is, at this level of analysis, intact. Nevertheless there are ~10 GH-containing RNA molecules per cell, as determined by "Northern" blot analysis. Cell-free translation in the reticulocyte system shows no translatable GH mRNA. After microinjection of genomic rat GH genes, immunoreactive GH protein is synthesized in these hybrids. We tentatively conclude that the failure to produce GH in these hybrids is due to lack of transcription of an intact GH gene.
3. Objective three: An image-intensification, computer-assisted method for analysis of differences in large cDNA populations has been developed. Use of this technology should allow comparison of any two RNA populations for specific changes following physiological or pharmacological stimuli. From this comparison, one will be able to detect specific increases or decreases in individual RNA species, without prior purification. One requirement for optimal use of this technology is a filter which allows the rapid reuse of attached clones of DNA in hybridizations, and we have developed such a method.
4. Objective four: We have purified human glucocorticoid receptors to about 50% homogeneity and immunized rabbits with this preparation. We now are characterizing these sera.
5. Objective five: We have obtained <sup>3</sup>H-labeled cortivazol and are using this radioligand in binding studies. We are establishing the toxicity limits of cortivazol in nude mice. We have conducted preliminary screens of the compound for efficacy against a variety of malignant and normal cells in tissue culture.

6. Objective six: We have employed the HeLa cell cell-free transcription system to study production of run-off transcripts from portions of the rat GH gene and have obtained transcription initiated ~ 30 b.p. downstream from a Goldberg-Hogness-like sequence 5'-ward from the first exon of the gene.
7. Objective seven: We have constructed vectors combining the probable promoter region of the GH gene with the pSVK vector, such that the GH promoter can be studied for its control over the transcription of E. coli galK gene. These plasmids contain, on a separate transcription unit, the E. coli xanthine-guanine phosphoribosyl transferase gene under control of the early SV40 promoter. The latter serves as an internal copy number standard as well as a means of positive selection when the entire vector has been internalized in host cells.
8. Objective eight: We have begun comparison of the glucocorticoid receptors of "New World" (squirrel) monkeys with those of "Old World" (cynomolgous) monkeys. These animals differ remarkably with respect to their circulating glucocorticoid levels, and this may be due to a difference in receptor affinity for steroid. We are attempting to label these receptors with the covalent affinity ligand for glucocorticoid receptor, dexamethasone mesylate.
9. Objective nine: A comparison of receptor occupancy and enzyme induction in HTC and FU55 rat hepatoma cells showed that tyrosine aminotransferase in FU55 only was fully induced by far below receptor-saturating concentrations of glucocorticoids. This surprising result indicated that in those cells either a secondary amplifying system for, or specially sensitive nuclear response to glucocorticoid-receptor complexes, occurs in those cells. This work was completed and terminated.

#### Proposed Course of Research

We propose to pursue the leads offered by the results listed above. An extensive discussion is precluded by limitations of space, but an outline of what we intend to do is as follows:

1. Examine our clone of lysis-resistant, receptor<sup>+</sup> CEM cells in somatic cell hybrids with r<sup>0</sup>, ract1 and r<sup>+</sup>, wild-type cells. We also will try again to map the human glucocorticoid receptor to a chromosome using CEM-C1 cells in hybrids with r<sup>0</sup> HTC cells.
2. Try to develop the cell-free transcription of GH DNA further, seeking interactions with steroids and their receptors.
3. Study the binding of purified glucocorticoid receptor to specific DNAs, such as MMIV, growth hormone and prolactin DNAs.
4. Attempt to cross-link receptor with DNA to see whether other non-receptor proteins also are involved in receptor-DNA interactions.
5. Continue to explore expression of GH and prolactin (Prl) genes in other cells by transfection experiments involving vectors of various sorts into cells and cell hybrids to study GH and Prl gene control.

6. Complete sufficient cell and animal experiments to bring cortivazol to phase I clinical trials (if these experiments warrant doing so). Define the nature of cortivazol binding in CEM cells.
7. Covalently label monkey glucocorticoid receptors with  $^3\text{H}$  steroid and study their physical properties.
8. Prepare  $> 90\%$  pure rat/human GRs and carry out partial amino acid analyses.
9. Covalently label and analyze the human and rat glucocorticoid receptors in steroid binding sites.
10. Prepare a cDNA library from CEM cells and use our computerized method to compare RNAs from steroid-treated and untreated cells to define the number of steroid-altered RNAs.
11. Study human leukemic cell receptors with anti-human antibody and mesylate labeling.
12. Complete restriction endonuclease analysis of Pr1 genes in the GH<sub>3</sub> x L cell hybrids.

#### Publications:

#### Original Papers

Harmon, J.M. and Thompson, E.B.: Isolation and characterization of dexamethasone-resistant mutants from the human lymphoid cell line CEM-C7. Mol. Cell. Biol. 1: 512-521, 1981.

Simons, S.S., Jr. and Thompson, E.B.: Dexamethasone-21-mesylate: an affinity label of glucocorticoid receptors from rat hepatoma tissue culture cells. Proc. Natl. Acad. Sci., USA 78: 3541-3545, 1981.

Harmon, J.M., Schmidt, T.J., and Thompson, E.B.: Non-glucocorticoid receptor-mediated effects of the potent glucocorticoid deacylcortivazol. Cancer Res. 42: 2110-2114, 1982.

Harmon, J.M. and Thompson, E.B.: Glutamine synthetase induction by glucocorticoids in the glucocorticoid-sensitive human leukemic cell line CEM-C7. J. Cell. Physiol. 110: 155-160, 1982.

#### Reviews

Thompson, E.B.: Factors affecting adrenocortical hormone function. Environ. Health Perspect. 38: 99-103, 1981.

Simons, S.S., Jr. and Thompson, E.B.: Affinity labeling of glucocorticoid receptors: new methods in affinity labeling. In Biochemical Actions of Hormones, Vol. IX, Litwack, G. (ed.), Academic Press, New York, 1982, pp. 221-254.

Thompson, E.B., Simons, S.S., and Harmon, J.M.: Deacylcortivazol, a potent glucocorticoid with unusual structure and unusual anti-leukemic cell activity. In Hormones and Cancer, Leavitt, W.W. (ed.), Plenum, New York, 1982, pp. 315-324.

Norman, M.R., Harmon, J.M., and Thompson, E.B.: The use of human cell culture systems for studying the action of glucocorticoids in human lymphoblastic leukaemias. In Hormones in Normal and Abnormal Human Tissues Vol. II, Fotherby, K. and Pal, S.B. (eds.), deGruyter, Berlin/New York, 1982, pp. 437-474.

Harmon, J.M., Schmidt, T.J., and Thompson, E.B.: Defective steroid receptors in a glucocorticoid resistant clone of a human leukemic cell line. In Hormones and Cancer, Leavitt, W.W. (ed.), Plenum, New York, 1982, pp. 301-313.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05214-11 LB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  DNA Synthesis in Mammalian Cells		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	S. H. Wilson	Medical Officer LB NCI
Other:	P. Becerra	Visiting Fellow LB NCI
	A. Hazra	Visiting Fellow LB NCI
	S. Detera	Visiting Fellow LB NCI
	E. Karawya	Visiting Fellow LB NCI
COOPERATING UNITS (if any)  John Minna, NCI		
LAB/BRANCH  Laboratory of Biochemistry		
SECTION  Biosynthesis Section		
INSTITUTE AND LOCATION  DCBD, NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  6	PROFESSIONAL:  5	OTHER:  1
CHECK APPROPRIATE BOX(ES)  <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) We continued an investigation of the <u>structure of mammalian DNA polymerase <math>\alpha</math></u> . Our present results emphasize the presence and abundance of a <u><math>\sim 120,000-M_r</math> <math>\alpha</math>-polymerase catalytic polypeptide</u> in mouse myeloma cells and in several other <u>mammalian cell types</u> . Catalytic polypeptides of other $M_r$ s also were observed in these cells, and it was found that two molecular forms of purified myeloma $\alpha$ -polymerase had different subunit compositions. Further, we observed that <u>in vitro translation of total calf thymus poly(A<sup>+</sup>) RNA resulted in synthesis of a <math>120,000-M_r</math> polypeptide with DNA polymerase activity</u> . A panel of monoclonal antibodies <u>against mammalian DNA polymerases <math>\alpha</math> and <math>\beta</math></u> were further characterized. Several lines of evidence were obtained that <u>one of these antibodies cross-reacts with <math>\alpha</math>-polymerase and <math>\beta</math>-polymerase</u> . These <u>antibodies are now well enough characterized for use in studies on DNA polymerase structure and gene cloning</u> . <u>Structure-function relationships of E. coli DNA polymerase I large fragment</u> were evaluated using <u>pyridoxal 5'-phosphate covalent modification of the enzyme</u> . Our data indicate that a <u>lysine residue in an essential binding site for dNTP</u> is a target for pyridoxal 5'-phosphate modification.		



## Project Description:

Objective: The ultimate objective of this research program is to understand mechanisms of DNA synthesis in mammalian cells. Our approach is the examination of DNA replication in vitro using purified DNA polymerases and other required proteins.

It is anticipated that these studies on the properties and specificities of the purified DNA replication proteins will, in conjunction with results from other workers, help answer important questions about mechanisms of DNA replication in the cell.

Methods Employed: Procedures have been developed for purification of DNA polymerases and DNA binding proteins from mouse and other tissues. These procedures involve subcellular fractionation, various types of ionic separation, gel filtration, and affinity chromatography, and assays for DNA polymerase activities using a variety of reaction conditions. Cell fractionation procedures and characterizations of reaction products were performed using conventional techniques of rate-zonal centrifugation, scintillation spectrophotometry, thin-layer chromatography, and gel electrophoresis.

## Major Findings:

### I. Mechanism of DNA Polymerase

We found that pyridoxal 5'-phosphate (PLP) is an inhibitor of DNA polymerase activity of *E. coli* DNA polymerase I large fragment. Kinetic studies indicated that PLP inhibition was noncompetitive with respect to dNTP, and Hill plot analysis to determine the molecular order of inhibition revealed a coefficient of 1.8. Reduction of the PLP-treated enzyme with [<sup>3</sup>H] sodium borohydride resulted in covalent incorporation of ~ 3 to 4 mol of PLP/mol of enzyme. This incorporation was at lysine residues, yielding ε-(5'phosphopyridoxyl) lysine. The PLP-modified enzyme had no DNA polymerase activity. The presence of dNTP during the modification reaction partially protected the enzyme from inactivation and blocked incorporation of 1 mol of PLP/mol of enzyme. The magnitude of this effect on PLP incorporation was independent of the presence of template-primer. These data suggest that a lysine residue in an essential binding site for dNTP was a target for PLP modification.

Knowledge of the primary structure location of this and the other two modified lysine residues will contribute to the identification of the active center in this DNA polymerase. To this end, we have observed that only one V8 protease peptide is protected from PLP modifications by dNTP and that a very similar result is obtained with CNBr peptides. The precise location of these peptides in the primary structure of the enzyme recently reported by Brown *et al.* is under investigation in collaboration with those workers.

### II. Structure of Mammalian DNA Polymerases

- a. Activity Gel Analysis. During the past year, because a new and particularly powerful assay method became available, we pursued further studies

on the structure of mammalian DNA polymerase  $\alpha$ . In this new assay, proteins in a sample containing a DNA polymerase are denatured and resolved by SDS-polyacrylamide gel electrophoresis. SDS is soaked from the gel, and polypeptides are allowed to remain in situ; then the intact gel is incubated in a DNA polymerase reaction mixture in order to localize the polymerase (Spanos et al., Nucleic Acids Res. 9, 1825-1839, 1981). Using this "activity gel" analysis we found that a crude homogenate of mouse myeloma contains a 40,000- $M_r$  polypeptide with strong DNA polymerase activity; from its  $M_r$  and catalytic properties this enzyme was identified as  $\beta$ -polymerase. The homogenate also contains two additional DNA polymerase activities giving relatively strong bands at  $M_r = \sim 76,000$  and  $\sim 120,000$ , respectively. Results on the catalytic properties of both of these enzymes indicate that they are  $\alpha$ -polymerases. Further evidence for the existence of a  $\sim 120,000$ - $M_r$   $\alpha$ -polymerase catalytic polypeptide came from the observation that a purified preparation of one of the recognized species of mouse myeloma  $\alpha$ -polymerase is composed of a  $\sim 120,000$ - $M_r$  polypeptide, as revealed by Coomassie blue staining after SDS-polyacrylamide gel electrophoresis in the presence of 3 M urea. This purified enzyme did not contain polypeptides in the 40,000- to 70,000- $M_r$  range and is capable of producing a strong band at 120,000- $M_r$  in the activity gel assay. These observations further support the concept discussed in last year's report that at least one species of mammalian  $\alpha$ -polymerase contains a  $\sim 120,000$ - $M_r$  catalytic polypeptide.

- b. Identification of Calf Thymus DNA Polymerase mRNA. DNA polymerase  $\alpha$  heterogeneity is well-recognized in many eukaryotic systems, yet analysis at the polypeptide level has failed to explain adequately functional and biochemical origins of this property. Among other approaches, we are studying this problem by attempting to isolate and characterize the  $\alpha$ -polymerase gene(s) and corresponding transcript(s). Our method for identification of  $\alpha$ -polymerase messenger RNA is to assay products of cell-free translation for catalytically active DNA polymerase polypeptides using in situ DNA synthesis after SDS-polyacrylamide gel electrophoresis (activity gel analysis). We have found that in vitro translation of calf thymus polyadenylated RNA results in synthesis of a 120,000- $M_r$  polypeptide with DNA polymerase activity; the  $M_r$  of this polypeptide is identical to that of the main  $\alpha$ -polymerase catalytic polypeptide in crude extracts from calf cells and in preparations of purified calf  $\alpha$ -polymerase. These results suggest that the calf thymus 120,000- $M_r$  catalytic polypeptide may be a primary translation product. In addition, these findings illustrate the utility of activity gel analysis for the identification of DNA polymerase messenger RNA.
- c. Monoclonal Antibodies Against Mammalian DNA Polymerases. Hybrid cell lines were produced by fusion of mouse myeloma cells to spleen cells from rats immunized with highly purified bovine DNA polymerase  $\alpha$ . Twenty-two stable hybridoma clonal lines were obtained that secreted monoclonal antibodies against components of the DNA polymerase preparation. One of these, MCPol-1, was chosen for further study. This clone was grown in ascites, and the secreted antibody was purified to homogeneity in mg amounts by fractional precipitation and Sephacryl S-200 gel filtration. This antibody was of the IgM class. Using "activity gel" methods to

identify and measure DNA polymerases, we found that the antibody was capable of immunoprecipitation of purified polymerases  $\alpha$  and  $\beta$ , and the antibody immunoprecipitated both of these DNA polymerases from crude extracts of bovine fibroblast. Using radioimmunoassay we observed that the antibody reacted with purified calf thymus  $\alpha$ -polymerase and  $\beta$ -polymerase. Similar observations of cross-reaction with the two DNA polymerases also were made by immunoblotting analysis after SDS-polyacrylamide gel electrophoresis. The presence of common antigenic determinants on these two polymerases also was suggested by results obtained with a polyclonal antibody raised against chicken  $\beta$ -polymerase.

Immunoprecipitation experiments with extracts from bovine cells labeled with  $^{35}\text{S}$ -methionine demonstrated specific reaction with a 40,000-M<sub>r</sub> polypeptide ( $\beta$ -polymerase) and other polypeptides, mainly of M<sub>r</sub> = 120,000 and 185,000.

### III. Studies on Putative DNA Polymerase Accessory Proteins

#### a. Properties of a Novel Oligonucleotide Releasing DNA Exonuclease.

Preparations of helix destabilizing protein-1 from mouse myeloma contain a novel DNA exonuclease. This enzyme can be separated from the helix destabilizing protein and obtained in highly purified form. A 41,000 M<sub>r</sub>-polypeptide is a main constituent of the purified enzyme, and this polypeptide comigrates with the exonuclease activity during the final step of the purification, Sephacryl S-200 gel filtration, where the enzyme has a native M<sub>r</sub> of 40,000. Overall purification of the enzyme activity is approximately 20,000-fold. This enzyme releases 5' oligonucleotides from ssDNA substrates, requires a divalent cation for activity and does not degrade dsDNA, closed circular ssDNA, or RNA. The enzyme does not require sulfhydryl reducing agents and is resistant to 1 mM N-ethylmaleimide. With 3' end labeled (pdA)<sub>55</sub> as substrate, >95% of the labeled products are (pdA)<sub>4</sub> and (pdA)<sub>5</sub> as revealed by electrophoresis in 23% polyacrylamide, 7 M urea gels; with 5' end labeled (pdA)<sub>55</sub> as substrate, the main labeled product is (pdA)<sub>2</sub>, but some release of (pdA)<sub>3</sub>, (pdA)<sub>4</sub>, (pdA)<sub>5</sub>, and (pdA)<sub>6</sub> occurs also. The rate of product release from these 3'-end and 5'-end labeled substrates is nearly identical at 37°C. With uniformly labeled (pdA)<sub>55</sub> as substrate, the size distribution of labeled products is equal to a combination of those observed with the 3'-end and 5'-end labeled substrates. We conclude that this enzyme acts in both the 3' → 5' and the 5' → 3' directions. The enzyme is similar to, but not identical with, the human placenta correxonuclease described by Doniger and Grossman (J. Biol. Chem. 251, 4579-4587, 1976).

### IV. Regulation of DNA Polymerase Alpha in Monkey Cells in Culture

In DNA collaboration with E.L. Kuff, we have continued an examination of levels of DNA polymerase activities in contact inhibited monkey cells in culture. The results of this study are still preliminary, and we anticipate detailed description of findings in a later report.

Significance to Cancer Research:

Detailed knowledge of the mechanisms of DNA synthesis is vital to our understanding the molecular biology of neoplasia. Many approaches are being used to investigate DNA synthesis in normal and neoplastic cells, and advances on all levels will prove useful in preventing, treating, and ultimately controlling cancer. Activity of DNA synthesizing proteins is necessary for maintenance of a rapid rate of cell division, and in some cases there is evidence that these proteins may play an important role in the development of the neoplastic state.

Proposed Course of Research:

1. To further characterize DNA replication proteins from mammalian cells and from vaccinia virus-infected cells.
2. To further study the enzymatic mechanism of DNA polymerases and the properties of single-stranded DNA specific binding proteins of interest.
3. To investigate activity of purified DNA replication proteins in vitro using as template either a single-stranded closed circular viral DNA, viral replication intermediates, homopolymer DNA, or vaccinia virus DNA.

Publications:

Albert, W., Grummt, F., Hübscher, U., and Wilson, S.H.: Structural homology among calf thymus  $\alpha$ -polymerase polypeptides. *Nucleic Acids Res.* 10:935-946, 1982.

Detera, S.D. and Wilson, S.H.: Studies on the mechanism of Escherichia coli DNA polymerase I large fragment. Nascent chain termination and modulation by polynucleotides. *J. Biol. Chem.*, in press, 1982.

Karawya, E.M. and Wilson, S.H.: Studies on catalytic subunits of mouse myeloma  $\alpha$ -polymerase. *J. Biol. Chem.*, in press, 1982.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05231-08 LB
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Role of Subunit Interactions in Enzyme Chemistry and Cellular Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: C.B. Klee Chief, Macromolecular Interactions Section LB NCI

Others:

M. Oldewurtel	Staff Fellow	LB NCI
M. Krinks	Chemist	LB NCI
J. Williams	Physical Science Technician	LB NCI
R. Miller	Technician	LB NCI
A. Manalan	Special Medical Staff Fellow	LB NCI
D. Newton	Research Chemist	LB NCI
A-M. Nunez	Visiting Fellow	LB NCI

COOPERATING UNITS (if any) Dr. J. Schiloach, NIAMDD; Dr. D. Takemoto, Kansas University; Mr. Richard Feldman, CR-CCB; Dr. P. Cohen, University of Dundee, Scotland; Dr. D. Hathaway, Indiana University; Dr. D. Flockhardt, Vanderbilt University.

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Macromolecular Interactions Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

6.5

PROFESSIONAL:

3

OTHER:

3.5

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) The interaction of calmodulin with its target proteins including cyclic nucleotide phosphodiesterase, adenylate cyclase, myosin kinase, phosphorylase kinase, cAMP dependent protein kinase, calcineurin and protein phosphatase has been studied in order to understand the mechanism of regulation of  $Ca^{2+}$ -dependent cellular processes by this protein. Binding of  $Ca^{2+}$  or  $Tb^{3+}$  to specific sites on calmodulin generates stepwise conformational transitions. The different  $Ca^{2+}$ -dependent interactions of calmodulin with its targets allow calmodulin to effect a kinetic regulation of the  $Ca^{2+}$  signal. In contrast to phosphorylase kinase which interacts with calmodulin in the absence of  $Ca^{2+}$ , phosphodiesterase needs  $Ca^{2+}$  for interaction. This enzyme exhibits a highly cooperative  $Ca^{2+}$  activation and different degrees of  $Ca^{2+}$  occupancy are needed for interaction and activation. Although calmodulin cannot interact simultaneously with more than one protein, different targets recognize and interact with different conformers of the regulatory protein. A third regulatory mechanism involves the interaction of the two second messengers,  $Ca^{2+}$  and cAMP. These are closely linked at the level of phosphorylation of the target proteins, regulation of cyclic nucleotide and  $Ca^{2+}$  concentrations and also by virtue of interactions between the regulatory subunit of protein kinase and calmodulin.



Project Description:Objectives:

To study the functional role of subunits and protein-protein interactions. The system under investigation is the  $\text{Ca}^{2+}$ -dependent regulation of enzymes mediated by calmodulin. Emphasis will be on cyclic nucleotide phosphodiesterase, enzymes regulating protein phosphorylation and their modulation by inhibitory proteins such as calcineurin and by the other second messenger, cAMP.

Methods Employed:

The project involves the study of properties of proteins, purified and analyzed by the usual techniques of protein isolation, including many types of chromatography and electrophoresis. The enzymes are studied by examination of their optical, hydrodynamic, and kinetic properties as well as by suitable chemical measurements and modifications. The role of these proteins in cellular regulation will be studied in tissue cultured cells.

Regulation of Cyclic Nucleotide Phosphodiesterase:

The  $\text{Ca}^{2+}$ -dependent regulation of several cellular processes is mediated by the ubiquitous, intracellular  $\text{Ca}^{2+}$ -binding protein, calmodulin. Interaction of  $\text{Ca}^{2+}$ -dependent cyclic nucleotide phosphodiesterase and calcineurin with calmodulin is studied as a model system to elucidate the mechanism of action of calmodulin. Methods developed for these systems are applied to the study of interaction of calmodulin with other enzymes in collaboration with other investigators.

The stimulus-response coupling mediated by  $\text{Ca}^{2+}$  involves several successive steps: (1) transitory increase in intracellular  $\text{Ca}^{2+}$  from  $10^{-7}$  to  $10^{-6}$ - $10^{-5}\text{M}$ , (2) interaction of  $\text{Ca}^{2+}$  with the  $\text{Ca}^{2+}$  receptor, calmodulin, (3) interaction of the activated calmodulin- $\text{Ca}^{2+}$  complex with the various target proteins and (4) coordinated activation of several enzymatic reactions. We previously reported that  $\text{Ca}^{2+}$  binding to calmodulin occurs in a stepwise fashion. The identity of the  $\text{Ca}^{2+}$  sites occupied sequentially by  $\text{Ca}^{2+}$  was tentatively identified on the basis of fluorescence energy transfer studies using  $\text{Tb}^{3+}$  as a ligand. Binding of two mol of  $\text{Tb}^{3+}$  or two mol of  $\text{Ca}^{2+}$  per mol of calmodulin induces an increased tyrosine fluorescence which reflects a change in the environment of both tyrosyl residues 99 and 138. Binding of two additional  $\text{Tb}^{3+}$  results in a quenching of the tyrosine fluorescence and in increased  $\text{Tb}^{3+}$  fluorescence. The tyrosyl residue involved in this apparent energy transfer has been identified as Tyr 99, since nitration of Tyr 99 significantly reduces the  $\text{Tb}^{3+}$  fluorescence. This result is compatible with a distance of  $5\text{\AA}$ , between Tyr 99 and the  $\text{Ca}^{2+}$  bound in the fourth  $\text{Ca}^{2+}$ -binding site, as calculated by computer fitting of the sequence of the COOH-terminal half of calmodulin (sites III and IV) to the coordinates of carp parvalbumin (in collaboration with R. Feldman). Tyr 138 is located more than  $10\text{\AA}$  away from sites III or IV. Assuming that  $\text{Ca}^{2+}$  and  $\text{Tb}^{3+}$  have similar relative affinities for the different  $\text{Ca}^{2+}$  sites, the third site to be occupied is site IV. This assignment is tentative since  $\text{Tb}^{3+}$

has a much higher affinity than  $\text{Ca}^{2+}$  for the cation-binding sites and although it is an activator of calmodulin-dependent phosphodiesterase at very low concentrations it is a strong inhibitor at concentrations above  $10^{-7}\text{M}$  (M. Epstein and M. Krinks).

The stepwise binding of  $\text{Ca}^{2+}$  or  $\text{Tb}^{3+}$  is accompanied by the formation of different conformers according to the degree of site occupancy. Calmodulin can thereby translate a quantitative  $\text{Ca}^{2+}$  signal into different cellular responses if different enzymes recognize different conformers. In order to test this hypothesis we prepared large batches of calmodulin (0.8 - 1.5g) and from these made calmodulin peptides by limited proteolysis with trypsin in EGTA or  $\text{Ca}^{2+}$ . Methods were developed to purify these peptides in large quantities by high performance liquid chromatography. Peptides 1-106, 1-90, 107-148, 1-77 and 78-148 have been isolated in 10-60% yield. None of the fragments stimulates  $\text{Ca}^{2+}$ -dependent cAMP phosphodiesterase when tested at concentrations up to  $10^{-6}\text{M}$ . Mixtures of peptides 1-90 or 1-106 with 77-148 are also inactive. Peptide 78-148 ( $> 10^{-6}\text{M}$ ) prevents the activation of phosphodiesterase by  $10^{-8}\text{M}$  calmodulin. None of the other fragments displays similar calmodulin antagonist activity. Interestingly the same peptide, 77-148, has previously been found to display weak calmodulin-like activity in the activation of phosphorylase kinase (Kuznicki et al., FEBS Lett. 130: 141-145, 1981). Thus, peptide 78-148 can behave as either a calmodulin agonist or antagonist depending upon the enzyme under study (D. Newton, J. Shiloach, M.H. Krinks, M.D. Oldewurtel and C.B. Klee). D. Newton is now testing the ability of these peptides to interact with the calmodulin antagonist, phenothiazines, and will thereby test the currently accepted hypothesis that phenothiazines interact with calmodulin at sites ordinarily recognized by its target enzymes. In addition, D. Newton has shown that phenothiazines also interact with the enzymes themselves and may recognize the calmodulin-binding site on the target proteins. These studies, combined with the analysis of the conformational changes which accompany binding of  $\text{Ca}^{2+}$  to peptides (by M. Oldewurtel described in the last report) will enable us to characterize, in some detail, the calmodulin interacting site(s) for its target proteins and antagonists.

A second part of our research consists of the study of the interaction of calmodulin with its target proteins. It has been carried out in the laboratory by M. Krinks, A. Manalan and D. Newton, as well as in collaborative projects with Dr. J. Shiloach (NIAMDD) and Dr. P. Cohen (University of Dundee, Scotland). Studies reported previously indicated that  $\text{Ca}^{2+}$ -calmodulin dependent phosphodiesterase is different from other forms of the enzyme ("low  $K_m$ ", cGMP stimulated and rod outer segment enzymes). On the other hand, the calmodulin-sensitive enzymes isolated from different tissues and different organisms appear to be very similar in primary structure. Small differences in subunit molecular weight are observed which are probably the result of limited proteolysis (M. Krinks and D. Takemoto). The largest extent of stimulation by calmodulin is observed with enzymes of high molecular weight. Efforts were therefore made to minimize proteolysis during the purification of the enzyme. A large scale, three-step, purification procedure has been developed yielding an enzyme which is a dimer of two identical subunits with molecular weights of 59,000. It is stimulated 15-25 fold by calmodulin and has a specific activity of 300-350 units/mg. M. Krinks

has been studying the activation of this enzyme by limited proteolysis with trypsin. Proteolysis of the enzyme-calmodulin complex occurs in at least two successive steps: a first cleavage yields a 52,000  $M_r$  polypeptide which has lost 50% of its stimulated activity but is still activated two fold by calmodulin as opposed to 18 fold for the parent enzyme; subsequent cleavages yield a 38,000  $M_r$  species which is not activated by calmodulin and is not retained on calmodulin-Sepharose columns but has a high specific activity of 250 units/mg. In the absence of calmodulin the 52,000  $M_r$  enzyme is rapidly converted to a 36,000 dalton,  $Ca^{2+}$ -independent, enzyme which is also unstable and does not accumulate. The native phosphodiesterase is therefore composed of two domains, a catalytic domain resistant to proteolysis, and a calmodulin-binding domain which exerts a regulatory, inhibitory effect on the catalytic domain and is protected against proteolysis in the presence of calmodulin. Several other calmodulin regulated enzymes which also interact with calmodulin through their catalytic subunit such as myosin light chain kinase and ( $Ca^{2+} + Mg^{2+}$ ) ATPase are also activated by limited proteolysis and may share a common calmodulin binding domain. We are presently trying to isolate such common structures.

A. Manalan has been investigating the interactions of another calmodulin target protein, calcineurin. Calcineurin is a major calmodulin binding protein in brain. A similar, but not identical protein, has been detected in small amounts in heart extracts. This protein is a heterodimer composed of subunits of molecular weight 61,000 and 15,000 usually associated with cyclic nucleotide phosphodiesterase during purification. The difficult separation of calcineurin from calmodulin-dependent cyclic nucleotide phosphodiesterase has been facilitated by addition of a chromatofocusing step to the purification scheme. During the course of purification and characterization, preparations of calcineurin were consistently found to contain protein phosphatase activity. Investigations conducted in collaboration with Dr. P. Cohen (University of Dundee) revealed that this phosphatase activity demonstrated substrate specificity identical to that exhibited by protein phosphatase 2B isolated from skeletal muscle by A. Stewart. It is  $Ca^{2+}$ -dependent protein phosphatase and it is further stimulated 10-fold by the addition of calmodulin. Protein phosphatase 2B-like activity was found to co-elute with calcineurin when a mixture of partially purified calmodulin-binding proteins from bovine brain was subjected to chromatofocusing and was also found in highly purified calcineurin preparations. Purified preparations of the skeletal muscle phosphatase 2B revealed a subunit composition by SDS polyacrylamide gel electrophoresis (61,000, 58,000 and 15,000) similar to that of calcineurin. These results suggest that calcineurin and protein phosphatase 2B are similar proteins although the possibility that phosphatase 2B activity is a trace contaminant in the calcineurin preparations cannot yet be excluded. Like other calmodulin-dependent enzymes, calcineurin is extremely sensitive to limited proteolysis with trypsin and appears to contain a calmodulin binding domain.

None of the enzymes studied to date can form ternary complexes with calmodulin (binding of one protein prevents binding of another protein). Although some of these proteins may share a common calmodulin binding domain, it is likely that there are some differences in the interacting sites since



the requirements for interaction are different for different target proteins. In contrast to most calmodulin-dependent enzymes, phosphorylase kinase interacts with calmodulin in the absence of  $\text{Ca}^{2+}$  but needs  $\text{Ca}^{2+}$  for subsequent activation. The  $\text{Ca}^{2+}$  dependence of activation is therefore identical to the  $\text{Ca}^{2+}$ -binding isotherm of calmodulin and activation should be controlled by the diffusion rate of  $\text{Ca}^{2+}$ . On the other hand, phosphodiesterase which requires three to four  $\text{Ca}^{2+}$  per mol of calmodulin for interaction as well as for activation exhibits a highly cooperative  $\text{Ca}^{2+}$ -dependent activation curve. The interaction and the activation of the enzyme may also require different degrees of  $\text{Ca}^{2+}$  occupancy since the deactivation upon removal of  $\text{Ca}^{2+}$  is very fast and results from partial removal of  $\text{Ca}^{2+}$  from the activated complex. These different modes of interaction enable calmodulin to regulate different enzymes to different extents and at different rates.

We have obtained evidence that calmodulin regulation of cytosolic enzymes can be modulated by other intracellular  $\text{Ca}^{2+}$ -binding proteins such as calcineurin as well as by cAMP-dependent phosphorylations. Conversely calmodulin and  $\text{Ca}^{2+}$  can control cAMP-dependent phosphorylations by acting directly or indirectly at the level of the cAMP-dependent protein kinase. In brain, a fraction of the Type II cAMP-dependent protein kinase interacts with calmodulin in a  $\text{Ca}^{2+}$ -dependent fashion. This interaction occurs at the level of the regulatory subunit of the kinase.

#### Significance to Biomedical Research and the Program of the Institute:

The proteins being studied are important examples of enzymes regulated by protein-protein interactions. cAMP phosphodiesterase, is one of the two enzymes responsible for the control of cAMP levels, which are critical for the regulation of cell growth and differentiation. The potential role of calmodulin in mediating the cellular effects of  $\text{Ca}^{2+}$  and the functional state of contractile and cytoskeleton proteins is of obvious importance. This ubiquitous regulatory protein also provides a link between cyclic nucleotide levels and  $\text{Ca}^{2+}$  regulation of cell functions. The ability of calmodulin to regulate a large number of biological processes represents a novel mechanism with great potential physiological importance.

#### Future Course of Research:

Calmodulin plays a unique role in the regulation of cellular processes mediated by cytosolic  $\text{Ca}^{2+}$ : that of a universal modulator of  $\text{Ca}^{2+}$  acting as a second messenger. It may also function as a coupling factor in the dual regulation of cellular processes by  $\text{Ca}^{2+}$  and cAMP. We will continue to study the structure of calmodulin in solution to characterize the multiple  $\text{Ca}^{2+}$  conformers responsible for multiple functions, and to identify the calmodulin interacting site(s) with its target proteins. We will attempt to isolate and characterize the interacting sites of the target proteins cyclic nucleotide phosphodiesterase and calcineurin. We will try to identify the link between the two second messengers,  $\text{Ca}^{2+}$  and cAMP, by studying the effect of calmodulin and  $\text{Ca}^{2+}$  on cAMP-dependent protein kinase and protein phosphatase. These studies will be carried out at the molecular level with purified and characterized proteins as well as at the cellular level with cells in tissue culture to

correlate the "in vitro" observations with physiological events. Among the model systems in which the role of calmodulin and other  $\text{Ca}^{2+}$  binding proteins will be investigated are: The differentiation of neuroblastoma glioma hybrid NG-108-15 and in vitro transformation of cells.

#### Publications:

1. Adelstein, R.S. and Klee, C.B.: Purification of smooth muscle myosin light chain kinase. In: Methods in Enzymology, Vol. 85, 1982, pp. 298-308.
2. Klee, C.B., Krinks, M.H., Hathaway, D.R. and Flockhardt, D.A.: Interaction of calmodulin with cAMP-dependent protein kinase in brain. In: Recent Advances in Calcium and Cell Functions, S. Kakiuchi and H. Hidaka (Eds.) Plenum Press, 1982, pp. 303-311.
3. Davies, P.J.A. and Klee, C.B.: Calmodulin-binding proteins: A high molecular weight calmodulin binding protein from bovine brain. Biochem. Internatl. 3: 203-212, 1981.
4. Stewart, A.A., Ingebritsen, T.S., Manalan, A., Klee, C.B. and Cohen, P.: Discovery of a  $\text{Ca}^{2+}$ -and calmodulin-dependent protein phosphatase. Probable identity with calcineurin (CaM-BP80). FEBS Lett. 137: 80-84, 1982.
5. Picton, C., Klee, C.B. and Cohen, P.: The regulation of muscle phosphorylase kinase by calcium ions, calmodulin and troponin C. Cell Calcium 2: 281-294, 1981.
6. Salter, R.S., Krinks, M.H., Klee, C.B. and Neer, E.J.: Calmodulin activates the isolated catalytic unit of brain adenylate cyclase. J. Biol. Chem. 256: 9830-9833, 1981.
7. Klee, C.B. and Vanaman, T.C.: Calmodulin. In: Advances in Protein Chemistry, 35, J.T. Edsall, F.M. Richards and C.B. Anfinsen, (Eds.) in press, 1982.
8. Oldewurtel, M.D., Krinks, M.H., Lee, J.W., Williams, W.F. and Klee, C.B.: Isolation and characterization of intracellular  $\text{Ca}^{2+}$  binding proteins by high performance liquid chromatography. Protides and Biological Fluids XXX Colloquium, Brussels, Pergamon Press, Oxford, May 3-6, 1982, in press.
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10. Adelstein, R.S. and Klee, C.B.: Purification and Characterization of Smooth Muscle Myosin Light Chain Kinase. J. Biol. Chem. 256: 7501-7509, 1981.
11. Hathaway, D.R., Adelstein, R.S. and Klee, C.B.: Interaction of Calmodulin with Myosin Light Chain Kinase and cAMP-dependent Kinase in Bovine Brain. J. Biol. Chem. 256: 8183-8189, 1981.
12. Barbehenn, E.K., Craine, J.E., Chrambach, A. and Klee, C.B.: Characterization of Polynucleotide Phosphorylase from M. luteus and Isolation of the 13,000 Base Poly(A) Product of the Polymerization Reaction. J. Biol. Chem. 257: 1007-1016, 1981.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05234-08 LB																				
PERIOD COVERED October 1, 1981 to September 30, 1982																						
TITLE OF PROJECT (80 characters or less)  Interrelations between the Genomes of SV40 and African Green Monkeys																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table border="0"> <tr> <td>PI:</td> <td>M.F. Singer</td> <td>Chief, Nucleic Acid Enzymology Section</td> <td>LB NCI</td> </tr> <tr> <td>Other:</td> <td>S. Lord</td> <td>Staff Fellow</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>S. Segal</td> <td>Expert</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>J. Saffer</td> <td>Staff Fellow</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>M. Lerman</td> <td>Visiting Scientist</td> <td>LB NCI</td> </tr> </table>			PI:	M.F. Singer	Chief, Nucleic Acid Enzymology Section	LB NCI	Other:	S. Lord	Staff Fellow	LB NCI		S. Segal	Expert	LB NCI		J. Saffer	Staff Fellow	LB NCI		M. Lerman	Visiting Scientist	LB NCI
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	M. Lerman	Visiting Scientist	LB NCI																			
COOPERATING UNITS (if any)  Dr. Paul Berg, Department of Biochemistry, Stanford University Medical School																						
LAB/BRANCH  Laboratory of Biochemistry																						
SECTION  Nucleic Acid Enzymology Section																						
INSTITUTE AND LOCATION  NCI, NIH, Bethesda, Maryland 20205																						
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SUMMARY OF WORK (200 words or less - underline keywords)  <p>Two distinct cloned segments of the African green monkey (<u>Cercopithecus aethiops</u>) genome that contain DNA sequences homologous to the control region of <u>simian virus 40</u> are being studied. The primary sequence of the homologous regions was <u>determined</u>. When included in vectors constructed by <u>molecular cloning</u> these sequences provide <u>transcriptional start sites</u>, as measured by expression of an <u>E. coli</u> gene in the vector. However the monkey segments do not provide the "<u>enhancer</u>" function that is present in the SV40 control region. Thus, in order for transcription to start within the monkey sequences, an enhancer segment must be separately incorporated into the vector. The monkey DNA sequences hybridize with cellular RNA indicating that they are normally transcribed. One of the sequences, which is 450 base pairs in length, is embedded in a genomic DNA region that is especially rich in interspersed repeated sequences and is hypersensitive to DNase I in monkey chromatin.</p>																						

## Project Description:

### Objectives:

A range of interactions occur between permissive cells and infecting viruses. Using simian virus 40 (SV40) as a model, we are studying one aspect of viral-host interaction, namely, the use of common regulatory components. When SV40 enters a permissive monkey cell it uses the host machinery for the initiation of early viral transcription and the synthesis of early viral proteins. Subsequently, interaction of an early viral protein (T-antigen) with the viral genome is required for viral DNA replication and the synthesis of late proteins. Replication too involves the use of a number of host enzymes. In addition to its effect on viral functions, T-antigen stimulates an increase in the level of the host enzymes required for DNA replication and host DNA replication itself. These events suggest that the viral and host DNAs might share similar sequences that are recognized by a variety of enzymes and regulatory proteins. Earlier we searched for, found and characterized DNA sequences in the monkey genome that are homologous to the control region of the SV40 genome. This region, a few hundred base pairs in length, includes the origin of DNA replication, the T-antigen binding sites, and the signals involved in the control of early viral transcription. We are now investigating the ability of the homologous monkey DNA segments to function in analogous ways.

### Methods Employed:

Standard tissue culture procedures are used. Other methods include radio-isotope tracer techniques, preparative and analytical ultracentrifugation, DNA-DNA and DNA-RNA hybridization both in solution and with DNA fixed to nitro-cellulose filters, column chromatography. Extensive use is made of restriction endonucleases and both preparative and analytical gel electrophoresis for the analysis and preparation of DNA fragments. Specific enzymatic procedures are used for modification or isotopic labeling of DNA fragments. Primary nucleotide sequence determination of DNA fragments is carried out by direct DNA sequencing techniques introduced by Maxam and Gilbert. DNA fragments are purified and prepared in  $\mu\text{g}$  quantities by recombinant DNA techniques using *E. coli* K12 cloning systems. The ability of cloned monkey segments to function as replication origins or sites for initiation of transcription is studied with special recombinant vectors designed for use in animal cells. The prototypical vector is pSV2, designed by Mulligan and Berg, this shuttle vector includes *E. coli* plasmid sequences, an *E. coli* gene (xanthine-guanine phosphoribosyl transferase, XGPRTase) and an SV40 control region. Molecular cloning allows replacing the SV40 control region with other DNA segments. In addition, DNA sequences can readily be introduced at various positions in the molecule. Desired constructions are made in vitro and then amplified in *E. coli* before transfection of mammalian cells. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research as approved by the NIH Biosafety Committee. All our work has been facilitated by extensive use of the NIH computer for storage and analysis of nucleotide sequence data. The mapping of RNA transcripts on DNA templates (including the constructed vectors) is done by the S1 nuclease technique and primer extension.

Major Findings:

In our earlier work a recombinant "library" of the DNA of the African green monkey (*Cercopithecus aethiops*) in a bacteriophage lambda vector was constructed. Using purified DNA fragments as probes, segments of monkey DNA homologous to the control region around the origin of replication of simian virus 40 (SV40) were isolated. Three distinctly different monkey segments homologous to the control region of SV40 were detected and characterized by subcloning and primary nucleotide sequence determination. Each segment is only a few hundred base pairs long and contains multiple and disconnected sequences homologous to the control region of SV40. The number and arrangement of the homologous sequences in each of the three segments differs and is distinct from the arrangement in the virus. The segments include homology to SV40 DNA regions known to be involved in the initiation of viral DNA replication and the start of early transcription. Specifically, the segments have the following features in common: (1) each contains multiple copies of the sequence GGGCGGPuPu, which also appears six times near the origin of SV40, (2) each contains several imperfect homologies to the central dyad symmetry of SV40, (3) each contains a long internal repeat, as does the origin region of SV40. Within the SV40 genome the GGGCGGPuPu repeats are known to be required for early transcription and to bind T-antigen. Recently, others have suggested that this region is a binding site for RNA polymerase II. The central dyad symmetry region also binds T- antigen, is equivalent to the origin of replication, and a portion of it is the start site for major early transcripts. The actual site of transcription initiation appears to be determined by the sequence TATAAT, 20-30 bp upstream from the start site. In the SV40 genome the TATAAT sequence is between the G-rich repeats and the start site. None of the 3 monkey sequences contains a TATAAT box.

We are testing the monkey sequences for their ability to replace the SV40 control sequences in both DNA replication and transcription initiation in animal cells. As described in last year's report, these experiments involve the use of a series of recombinant vectors constructed by Mulligan and Berg. At that time preliminary experiments had been carried out in collaboration with Subramani and Berg at Stanford using one of the cloned monkey segments, called clone 9. Those experiments have been completed. During this last year the properties of a second of the three cloned monkey segments, clone 7, have been investigated in Bethesda.

In brief, the results with clone 9 showed that the monkey segment does not support DNA replication in either the presence or absence of T-antigen. However, the monkey segment does provide transcriptional start sites as indicated by 1) the expression of the bacterial XGPRTase placed downstream from the monkey sequence and 2) the presence of messenger RNA for XGPRTase. Using S1 nuclease, specific transcriptional start sites in the monkey DNA segment have been mapped but thus far no distinctive nucleotide sequence appears to be associated with these start sites. Most significantly, the initiation of transcription within the monkey sequence depends on the presence, somewhere in the vector molecule, of a 72 bp long segment from the SV40 control region. By itself, this 72 bp segment does not permit transcription of XGPRTase.

The work with clone 7 has included several approaches. We have found that the 450 bp SV40-like region in clone 7 comprises a site that is hypersensitive to DNase I within monkey chromatin, as expected for a transcribed region. Furthermore, cellular RNA hybridizes to this cloned region suggesting that it is indeed transcribed. Like the segment in clone 9 it does not support DNA replication either in the presence or absence of T-antigen, when included in a constructed vector and transfected into monkey cells. Again, like the segment in clone 9, it supports the initiation of transcription of the *E. coli* XGPRTase gene when placed upstream from the gene, but only when the 72 base pair SV40 "enhancer" sequence is present on the vector. Within monkey genomic DNA, the 450 bp SV40-like region in clone 7 is located in a 17 kilobase pair long stretch that is remarkably rich in multiple different families of interspersed repeated sequences, including several members of the Alu family, and members of at least two other, previously undescribed families.

#### Significance to Cancer Research:

Our studies deal with the interactions between the viral genome and the genome of a permissive host. These studies are pertinent to the nature and mechanism of viral-host DNA interaction in permissive and transforming (oncogenic) infections. Further, it is now widely recognized that the genomes of tumor viruses contain sequences homologous to normal host DNA sequences. In the case of RNA tumor viruses these are oncogenes. Our experiments indicate that with some DNA tumor viruses such as SV40, regulatory sequences rather than coding sequences are shared by virus and host.

#### Proposed Course of the Research:

In the coming year we will complete the mapping of the transcripts initiated in vivo from the SV40-like region in clone 7 when present in constructed vectors. Then we will correlate the results with those obtained with clone 9 to try to identify the sequences that serve as binding sites and transcription initiation sites for RNA polymerase II. We also plan to study the transcription of this and neighboring sequences in cells that are not transfected with constructed vectors. In particular and consistent with our original objectives, we shall ask what effect SV40 infection has on transcription of this region. Our aim will be to find out whether SV40 T-antigen effects gene expression from this region. In addition, efforts are underway to establish an in vitro system for the study of messenger RNA splicing. Unspliced early SV40 RNA will be prepared and used as a splicing substrate with fractions of undifferentiated and differentiated teratocarcinoma cells.

#### Publication:

Queen, C., Lord, S.T., McCutchan, T.F. and Singer, M.F.: Three segments from the monkey genome that hybridize to SV40 have common structural elements. Mol. Cell. Biol. 1: 1061-1068, 1981.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05244-05 LB																				
PERIOD COVERED October 1, 1981 to September 30, 1982																						
TITLE OF PROJECT (80 characters or less) Organization of Repeated DNA Sequences in African Green Monkeys																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																						
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SUMMARY OF WORK (200 words or less - underline keywords) <p>The <u>highly repeated DNA</u> segments in the genome of the <u>African green monkey</u> (<u>Cercopithecus aethiops</u>) are being studied. These sequences can be classified in three groups on the basis of known structural features: tandem repetitions localized to centromeres, called <u>satellites</u>, short interspersed repeated sequences and long interspersed repeated sequences. A new monkey satellite (deca-satellite) with a ten base pair repeat unit has been found joined to another satellite. Deca-satellite is remarkable for its extensive polymorphism from one individual to another in the species. Multiple repeated sequence families have been shown to be dispersed within the monkey genome. Each family includes tens of thousands of copies of sequences several hundred base pairs long. Several of these may be <u>moveable elements</u> since they are flanked by short repeats of the region of the genome into which they are inserted.</p>																						



## Project Description:

### Objectives:

The detailed structure, organization and function of the highly repeated DNA sequences in the African green monkey (*Cercopithecus aethiops*) are being studied. These sequences fall into three groups: satellites are long tandemly repeated segments generally concentrated at centromeres, SINES are short repeated segments dispersed throughout the genome, LINES are long repeated segments also dispersed throughout the genome. We are interested in characterizing the various families of such sequences, establishing their frequencies within the genome and their presence in related primate genomes. Recently it has become evident that many of these sequences are changeable in position within DNA and also undergo reactions which bring the sequences of the various family members into conformity within a given genome. We are interested in the timecourse and frequency of such changes in order to define the fluidity of primate genomes. Another concern is the mechanisms by which movement and sequence change occur. Finally, we are interested in the function played by such sequences.

### Methods Employed:

DNA is isolated from fresh frozen tissue and from cells grown in tissue culture and purified by standard procedures. Structural analysis includes the use of a variety of enzymes, including restriction endonucleases, direct DNA sequencing methods (chemical procedure of Maxam and Gilbert), radioisotope tracers, gel electrophoresis, and centrifugation. A variety of nucleic acid hybridization techniques, both in solution and on nitrocellulose are used. DNA fragments are purified and amplified by molecular cloning in *E. coli* K12 host-vector systems. Genomic organization is studied by blotting and hybridization. Functional aspects focus first on transcription of these sequences both in vivo and in vitro. Finally, recombinant DNA vectors are constructed and transfected into animal cells to study expression. All recombinant DNA experiments are carried out under conditions required by the NIH Guidelines for Recombinant DNA Research as approved by the NIH Biosafety Committee. Somatic cell hybrids are constructed by standard techniques.

### Major Findings:

Some time ago we decided to study DNA segments in which  $\alpha$ -satellite, the major monkey satellite DNA (20% of genome) is joined to other DNA segments. For this purpose we constructed a library of the monkey genome in the bacteriophage lambda. Segments of the monkey genome between 15,000 and 20,000 base pairs long were generated by cleavage with EcoRI under a conditions designed to randomize most of the monkey genome. These segments were then linked to the arms of the lambda vector Charon4A and packaged, in vitro, into phage particles. The phage were then amplified in a manner designed to enhance the likelihood that the final collection of phage would represent most of the original recombinants. The library was screened for plaques that hybridize with  $\alpha$ -satellite. Approximately 0.7 percent of the plaques gave a strong hybridization signal with a  $^{32}\text{P}$ -labeled  $\alpha$ -satellite probe. On a random basis, and considering that about 15 to 20% of the total genome is  $\alpha$ -satellite, it

can be calculated that the expected percent would be of the order of 15. The discrepancy between what was observed and the calculated value is consistent with the assumption that the very long tandem stretches of  $\alpha$ -satellite that contain no EcoRI site at all would not occur in the library; such stretches must include almost 90% of the total  $\alpha$ -satellite in arrays containing 100 or more copies of the 172 base pair monomeric unit.

Seventeen phage were chosen at random from among those in the library that hybridized with  $\alpha$ -satellite and have been characterized to varying extents. Fifteen of the phage contain sequences other than  $\alpha$ -satellite sequences and thus represent genomic junctions between satellite and other sequences. We have found that there is a much greater amount of sequence divergence in the  $\alpha$ -satellite sequences joined to non- $\alpha$ -component, than that found in the average sequence characterized from bulk DNA. This finding is consistent with the predictions of the unequal crossing-over model for satellite evolution.

We have characterized four different types of sequences that join the  $\alpha$ -satellite. First, the previously known SINE family called ALU, was found amidst the satellite. Seven of the 17 phage contain ALU segments. This frequency is less than expected from a random distribution since 75% of the total phage in the library hybridized with ALU. From these phage as well as others containing distinct cloned portions of the monkey genome we could calculate that one ALU segment occurs every 8 kilobase pairs, on the average, in the monkey genome. Two cloned monkey ALU segments were sequenced. They have a structure closely homologous to the consensus sequence for ALU in humans (about 300 base pairs in length) as determined by others. As found with human ALUs, the monkey ALUs were flanked by short direct repeated sequences. This characteristic led us and others to suggest that ALU might be moveable in primate genomes, since such flanking direct repeats are typical of well characterized moveable elements in prokaryotes and eukaryotes. In the latter cases it is known that the duplications are reiterations of a sequence present only once before the entry of the moveable element into a given position. However finding such "empty" and "filled" sites for a ubiquitous primate system seemed hopeless. The  $\alpha$ -satellite cloned segments containing ALU afforded an unexpected opportunity to test this "target site duplication" hypothesis. One such phage was studied in detail. A single ALU family member was found to interrupt the  $\alpha$ -satellite sequence. By sequencing a 660 base pair long subcloned segment from the phage we demonstrated that the ALU is immediately flanked by 13 base pair long duplications of the known sequence of the satellite (some years ago the satellite was sequenced in this laboratory) at the point of insertion. These observations provide strong support for the hypothesis that ALU family members are moveable elements.

Three of the phage contained a common DNA sequence joined to  $\alpha$ -satellite. In one case, this sequence interrupted tandem arrays of satellite. We have now characterized these sequences and shown them to be members of a family of LINES. We call this family the Kpn-LINE family. Kpn-LINES are interspersed in both the monkey and the human genomes and repeated on the order of several thousand times. The longest cloned member of the family in our collection amounts to 6.3 kilobase pairs interrupting  $\alpha$ -satellite. Using subcloned portions we have demonstrated that at least 5.3 kilobase pairs of this unit are all repeated in both the monkey and human genomes. The data also suggest that the individual portions frequently occur as long units like the one interrupting the satellite. Furthermore, the majority of the family members in the two genomes, although showing similar

overall sequence organization, are species specific as detected by restriction endonuclease polymorphisms. Our results suggest that the Kpn-family may comprise a moveable primate family, since one member interrupts satellite. Also, the family members appear to be "homogenized" to a form characteristic of the species.

In one of the phage, the  $\alpha$ -satellite sequence is interrupted by about 800 base pairs of still another repeated sequence. This segment hybridizes with about 16% of a monkey library and is bounded on both sides by direct repeats of a short region of the interrupted satellite. Thus this newly discovered sequence also appears to be a moveable element.

Three other phage of the original 17 contain junctions between  $\alpha$ -satellite and a previously undescribed monkey satellite we named deca-satellite. Sub-cloning and sequence analysis showed that deca-satellite has a ten base pair repeat unit; the consensus sequence is 5'-AAACCGGNTC. The deca-satellite sequences are in the middle repeated class of genomic DNA. We investigated the overall organization of deca-satellite in the monkey genome by analyzing the pattern of bands that hybridize to a cloned deca-satellite probe, when genomic DNA is digested with a variety of restriction endonucleases. Surprisingly, the band pattern is highly polymorphic from one individual monkey to another. On the other hand, DNA from different tissues of a single individual give indistinguishable bands. We have initiated studies on families of African green monkeys in order to learn more about the extent of polymorphism and the rate at which the deca-satellite is reorganized in these animals. We have also shown that, in spite of the regular occurrence of the sequence 5'CCGG in the deca-satellite repeat unit, there is no obvious pattern of methylation of the C residues, nor are most of the C residues even methylated. In situ hybridization to intact metaphase monkey chromosomes was carried out with a  $^3\text{H}$ -labeled deca-satellite probe. Grains were observed on 26 out of the 60 typical chromosomes. All grains were localized at or near the centromeric regions.

We are also interested in whether the distribution of distinct forms of the various repeated sequences have specific localizations on monkey chromosomes. As reported last year, we constructed, for this purpose, a monkey/mouse somatic cell hybrid containing a single monkey chromosome. Unlike the mouse cells, the hybrid cells contain DNA that hybridizes with the  $\alpha$ -satellite DNA of the monkey. The presence of a single  $\alpha$ -satellite containing monkey chromosome was demonstrated by Giemsa-11 staining and by the absence of both this chromosome and monkey  $\alpha$ -satellite DNA sequences in cells after back-selection in bromodeoxyuridine. Hybridization of restriction endonuclease digested hybrid cell DNA with a cloned segment of African green monkey  $\alpha$ -satellite DNA showed distinctly different patterns from those observed with total monkey DNA. In particular, EcoRI and HaeIII restriction endonuclease sites are much more abundant in the satellite sequences in the thymidine kinase carrying chromosome than they are in total satellite. This result is consistent with the clustering of particular satellite variants on specific chromosomes. A library of hybrid DNA was constructed in a  $\lambda$  bacteriophage. Analyses of purified recombinant phage that hybridized with  $\alpha$ -satellite also indicated an abundance of EcoRI and HaeIII sites. Of 9 phage studied in detail, no two showed identical distributions of the two restriction sites in the  $\alpha$ -satellite sequences, suggesting the independent evolution of different domains within the single chromosome. Thus, the structure of the satellite is more complex than earlier experiments indicated.



Significance to Cancer Research:

Cancer research has suffered from a lack of basic knowledge about the eukaryote genome at the molecular level. Methods developed during the last few years have already demonstrated their power to deal with this vast and important unknown. The work we are doing is part of the broad effort to apply new approaches to the elucidation of complex genomes. In particular, we are concentrating on the surprisingly large amount of primate DNA included in the highly repeated sequences of yet unknown function. Substantial portions of these sequences appear to be moveable although on an unknown time scale. Nevertheless, the fluidity of genomes implied by these results gives a profound new insight into the nature of DNA. With increased understanding of DNA organization and fluidity, especially in primates, existing information and new data on neoplastic disease may become more amenable to rigorous analysis and understanding.

Future Course of the Research:

1. We will continue efforts to define the organization of DNA sequences at the ends of Kpn-LINE family members in order to test the hypothesis that the whole 6.3 kilobase pair segment may be a moveable element. Also, the nature of the set of Kpn-LINE family members on a single chromosome will be investigated. We will try to devise systems to study possible functions for this family.
2. The newly discovered repeated sequence family will be characterized further. Transcription of this sequence will be studied both in vivo and in vitro. Further, another recently detected abundant sequence family will be characterized.
3. The organization and fluidity of the deca-satellite will be further investigated.

Publications:

1. Grimaldi, G., Queen, C. and Singer, M.F.: Interspersed repeated sequences in the African green monkey genome that are homologous to the human Alu family. Nucleic Acids Res. 9: 5553-5568, 1981.
2. Singer, M.F.: Highly repeated sequences in mammalian genomes. Int. Rev. Cytol. 76: 67-112, 1982.
3. McCutchan, T., Hsu, H., Thayer, R.E. and Singer, M.F.: Organization of African green monkey DNA at junctions between  $\alpha$ -satellite and other DNA sequences. J. Mol. Biol. 157: 195-211, 1982.
4. Grimaldi, G. and Singer, M.F.: A monkey Alu-sequence is flanked by 13 base pair direct repeats of an interrupted  $\alpha$ -satellite DNA sequence. Proc. Natl. Acad. Sci. USA 79: 1497-1500, 1982.
5. Singer, M.F.: SINES and LINES: Highly repeated short and long interspersed sequences in mammalian genomes. Cell, Minireview, 28: 433-434, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05251-04 LB																
PERIOD COVERED      October 1, 1981 to September 30, 1982																		
TITLE OF PROJECT (80 characters or less)  <div style="text-align: center;">Structure and Transcription of the Ribosomal Genes in <u>Drosophila melanogaster</u></div>																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 40%;">I.B. Dawid</td> <td style="width: 30%;">Chief, Developmental Biochemistry Section</td> <td style="width: 10%;">LB NCI</td> </tr> <tr> <td>Other:</td> <td>R.K. Mandal</td> <td>Visiting Scientist</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>M.L. Rebbert</td> <td>Chemist</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>B. Wood</td> <td>Lab. Worker</td> <td>LB NCI</td> </tr> </table>			PI:	I.B. Dawid	Chief, Developmental Biochemistry Section	LB NCI	Other:	R.K. Mandal	Visiting Scientist	LB NCI		M.L. Rebbert	Chemist	LB NCI		B. Wood	Lab. Worker	LB NCI
PI:	I.B. Dawid	Chief, Developmental Biochemistry Section	LB NCI															
Other:	R.K. Mandal	Visiting Scientist	LB NCI															
	M.L. Rebbert	Chemist	LB NCI															
	B. Wood	Lab. Worker	LB NCI															
COOPERATING UNITS (if any)  <div style="text-align: center;">None</div>																		
LAB/BRANCH  <div style="text-align: center;">Laboratory of Biochemistry</div>																		
SECTION  <div style="text-align: center;">Developmental Biochemistry Section</div>																		
INSTITUTE AND LOCATION  <div style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</div>																		
TOTAL MANYEARS: <div style="text-align: center;">2.0</div>	PROFESSIONAL: <div style="text-align: center;">1.0</div>	OTHER: <div style="text-align: center;">1.0</div>																
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>The expression of <u>rRNA genes</u> from <u>Drosophila</u> is being studied with the primary aim of understanding the cause for the inactivity of interrupted genes. <u>Run-off transcription</u> in isolated nuclei of <u>Drosophila</u> cells showed that insertions are not transcribed in vitro; thus isolated nuclei preserve in vivo control in this respect. <u>Polymerase loading</u> on regions upstream and downstream of the <u>insertion</u> was measured, leading to the suggestion that interrupted rRNA genes are transcribed up to the point of insertion but not beyond. Preliminary experiments were carried out towards studying <u>rDNA transcription</u> in a <u>reconstituted system</u>.</p>																		



## Project Description:

### Objectives:

Ribosomal RNA genes are of interest because they encode the most abundant RNA molecules in every cell. Drosophila rRNA genes further have the special feature that some of the genes are continuous while others are interrupted by type 1 or type 2 insertions. We would like to understand the evolutionary origins and functional implications of ribosomal insertions in this organism. Further, we propose to study the functional requirements of rDNA expression. We have analyzed the transcription of rRNA genes in isolated nuclei of Drosophila cells with the aim of understanding the repression of expression of interrupted genes.

### Methods Employed:

Drosophila cells of the Schneider line were cultured and nuclei were prepared by detergent lysis. Embryo nuclei were isolated by homogenization and differential centrifugation. Nuclei were incubated in vitro in the presence of labeled nucleoside triphosphates and the RNA was isolated after various times. This RNA was hybridized to restriction fragments corresponding to various regions of interrupted and uninterrupted rRNA genes.

Oocytes were excised from Xenopus laevis and DNA was injected into their nuclei by a modification of the method introduced by Gurdon. Radioactive nucleoside triphosphate was also injected in some experiments. After incubation RNA was extracted and analyzed either by gel electrophoresis of labeled RNA or by S1 nuclease mapping using unlabeled RNA and suitably endlabeled restriction fragments.

### Major Findings:

As reported last year isolated nuclei from Drosophila cells incorporate nucleotides into pre-initiated RNA chains. Under these conditions the rRNA gene regions are effectively transcribed and some transcription is also found to originate from the spacer regions. The latter transcripts very likely are due to occasional read-through of transcripts at the natural termination site. In contrast, insertion sequences are not transcribed in the isolated nuclei, in agreement with earlier work in vivo. Using this nuclear system we tested whether RNA chains are loaded onto the interrupted rRNA genes upstream of the insertion. The results indicate that such regions are in fact loaded both in Schneider cells and in embryos. The inactivity of interrupted rRNA genes may therefore be due to termination of transcription at the point of insertion.

With the aim of developing a reconstructed system for the transcription of rRNA genes from Drosophila we injected such genes into frog oocyte nuclei. As a control, 5S RNA genes from Xenopus were effectively transcribed. No specific transcription was observed, in accord with other observations suggesting species specificity in rDNA transcription. Attempts continue to supplement the system with a homologous extract from Drosophila.

Significance to Cancer Research:

The regulation of expression of interrupted genes is a basic question of cellular metabolism in all cells. Likewise, the structure and function of rRNA genes is of basic and general importance for normal and malignant cells. The suggestion that ribosomal insertions may have arisen by the introduction of transposable elements is also of significance for an understanding of the possible role of such elements in genomic rearrangements and the generation of new regulatory connections.

Proposed Course of Research:

We shall inject a transcriptionally active extract from Drosophila together with rDNA into frog oocytes to attempt transcription of complete rRNA genes. The extract itself initiates accurately but does not distinguish interrupted from uninterrupted genes. We shall introduce intact and interrupted rRNA genes into Drosophila cells and study their transcription in vivo. To distinguish the products from natural rRNA we are constructing slightly modified rRNA genes.

Publications:

Long, E.O., Rebbert, M.L. and Dawid, I.B.: Structure and expression of ribosomal RNA genes of Drosophila melanogaster interrupted by type 2 insertion. Cold Spring Harbor Symp. Quant. Biol. 45: 667-672, 1981.

Long, E.O., Collins, M., Kiefer, B.I. and Dawid, I.B.: Expression of the ribosomal DNA insertions in bobbed mutants of Drosophila melanogaster. Molec. Gen. Genet. 182: 377-384, 1981.

Dawid, I.B. and Rebbert, M.L.: Nucleotide sequences at the boundaries between gene and insertion regions in the rDNA of Drosophila melanogaster. Nucleic Acids Res. 9: 5011-5020, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05252-04 LB																				
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>																						
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Sequence Organization in the Genome of <u>Drosophila melanogaster</u></p>																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 40%;">I.B. Dawid</td> <td style="width: 20%;">Chief, Developmental Biochemistry Section</td> <td style="width: 10%;">LB NCI</td> </tr> <tr> <td>Other:</td> <td>P.P. DiNocera</td> <td>Visiting Associate</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>M.E. Digan</td> <td>Guest Worker</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>M.L. Rebbert</td> <td>Chemist</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>B. Wood</td> <td>Lab. Worker</td> <td>LB NCI</td> </tr> </table>			PI:	I.B. Dawid	Chief, Developmental Biochemistry Section	LB NCI	Other:	P.P. DiNocera	Visiting Associate	LB NCI		M.E. Digan	Guest Worker	LB NCI		M.L. Rebbert	Chemist	LB NCI		B. Wood	Lab. Worker	LB NCI
PI:	I.B. Dawid	Chief, Developmental Biochemistry Section	LB NCI																			
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	M.E. Digan	Guest Worker	LB NCI																			
	M.L. Rebbert	Chemist	LB NCI																			
	B. Wood	Lab. Worker	LB NCI																			
COOPERATING UNITS (if any) Peter Wellauer, Swiss Institute for Experimental Cancer Research, Lausanne; Karl Illmensee, University of Geneva; Dr. M. Gans, CNRS, Gif-sur-Yvette, France																						
LAB/BRANCH <p style="text-align: center;">Laboratory of Biochemistry</p>																						
SECTION <p style="text-align: center;">Developmental Biochemistry Section</p>																						
INSTITUTE AND LOCATION <p style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</p>																						
TOTAL MANYEARS: <p style="text-align: center;">2.8</p>	PROFESSIONAL: <p style="text-align: center;">2.3</p>	OTHER: <p style="text-align: center;">0.5</p>																				
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																						
SUMMARY OF WORK (200 words or less - underline keywords) <p>             The structure of the transposable DNA called the F element has been studied in <u>Drosophila melanogaster</u>. Three DNA regions containing F elements were isolated from one stock of <u>D. melanogaster</u> and the boundaries of the F element were sequenced. The corresponding regions were isolated and partially sequenced from a different stock of <u>D. melanogaster</u>; the F element was absent from these regions. The comparison of the filled and empty sites showed that F element insertion is accompanied by <u>target site duplications</u> of variable length.           </p> <p>             A <u>female sterile mutation</u> that leads to <u>homeotic transformations</u> in <u>Drosophila</u> is being studied. We propose to isolate the gene by DNA cloning. To this end we have obtained DNA probes from the chromosomal region where the gene resides and have initiated a "<u>chromosomal walk</u>".           </p> <p>             To achieve <u>transformation</u> in <u>Drosophila</u> we have constructed plasmids in which the alcohol dehydrogenase gene has been inserted within <u>transposable elements</u>. These plasmids will be injected into <u>Drosophila</u> embryos by our collaborators in Switzerland.           </p>																						

Project Description:Objectives:

Drosophila melanogaster is an excellent model for the study of gene arrangement and expression because of its small genome, and the accessibility of the organism to genetic and cytogenetic techniques. We are presently studying the arrangement and expression of a new class of transposable sequences in this organism.

Dr. Gans and coworkers have characterized the effects of a temperature-sensitive maternal effect mutation of Drosophila melanogaster, fs(1)h, on development. Progeny descended from females homozygous or hemizygous for fs(1)h show segmental abnormalities which include missing appendages or appendages resembling those derived from more anterior segments. We are attempting to determine the molecular nature of this mutation.

Experiments were initiated with the aim to transform Drosophila by injection of DNA into embryos.

Methods Employed:

This project employs recombinant DNA methods to isolate different DNA molecules from D. melanogaster. We have prepared a set of clones which contain transposable elements. These elements are being used to determine the nucleotide sequence of selected regions by the Maxam/Gilbert procedure. We also use the cloned transposable elements to select additional homologous DNA molecules from a library of Drosophila DNA in lambda vectors.

In the study of mutant fs(1)h we employ libraries of Drosophila DNA in bacteriophage lambda. Using a probe from region 7D5,6 that was obtained from G. Rubin, Carnegie Institution, we select recombinant phage that proceed distally, toward the fs(1)h gene. Overlapping DNA molecules are identified by restriction mapping and cross-hybridization. Two stocks with chromosomal breakpoints in the region of interest have been obtained. The location of these breakpoints in the cloned DNA is being sought by Southern blotting.

Major Findings:

The family of transposable F elements in Drosophila melanogaster has been characterized. Three recombinant lambda phage were obtained from a library of DNA from strain Oregon R; F elements were embedded in different single-copy DNA in these phages. The boundary regions were sequenced, and the single-copy flanking DNA was used to isolate the corresponding regions of DNA from a library of DNA from strain Canton S. F elements were absent from the Canton S clones (Canton S contains F elements, but at other locations). The corresponding regions in the Canton S DNA were also sequenced. From these data we conclude that F elements are mobile sequences of a length of 4.8 kb, do not contain terminal repeats, lead to target site duplications between 8 and 13 nucleotides, and carry a stretch of about 20 A residues at one end. A canonical polyadenylation sites is present about 20 bp upstream from the oligo(A) stretch. This result may suggest that transposition of F elements could proceed by way of a RNA intermediate.



The mutation fs(1)h is being studied by isolating DNA from the region in which the gene is known to reside. At the present time, identification of functional units within cloned chromosomal fragments is dependent upon association of a gene mutant in the function with gross chromosomal rearrangement, such as inversion or translocation. We have identified the orientation of the original probe with respect to the centromere by demonstrating that we have crossed the proximal breakpoint of Df(1)sn. From the initial probe, we have proceeded approximately 30 kb toward the gene; we estimate that we are at most 100 kb from fs(1)h. We plan to use the breakpoints of two chromosomally rearranged alleles of fs(1)h to identify fragments of DNA surrounding the fs(1)h<sup>+</sup> gene.

#### Significance to Cancer Research:

Inasmuch as cancer is thought to involve a failure of basic cellular regulatory processes we need to understand in much more detail the structural basis and molecular mechanism of these processes. The organization and expression of the genome in animal cells is a subject of central importance in the analysis of regulatory processes during growth, development and evolution. Drosophila melanogaster is a particularly suited organism for the study of genome organization and function. The presence of transposable elements in the animal genome is an important recent finding. These elements may have great significance in the evolution of animal genomes and in changes that may alter major regulatory pathways. Drosophila is also particularly suitable for the study of developmental regulatory mutations. Genes like the fs(1)h gene may be of fundamental importance in regulating the normal development of cells and tissue.

#### Proposed Course of Research:

The study of transposable elements in Drosophila will proceed to characterize additional examples, and to use transposable elements in experiments designed to transform Drosophila by DNA injection. The mutant fs(1)h will be studied by cloning of DNA from the 7D region of the chromosome and identification of the gene with the help of rearrangements. Further, hybrid dysgenesis derived alleles are being sought that should assist gene isolation and identification.

#### Publications:

Pardue, M.L. and Dawid, I.B.: Chromosomal locations of two DNA segments that flank ribosomal insertion-like sequences in Drosophila: Flanking sequences are mobile elements. Chromosoma 83: 29-43, 1981.

Dawid, I.B., Long, E.O., DiNocera, P.P. and Pardue, M.L.: Ribosomal insertion-like elements in Drosophila melanogaster are interspersed with mobile sequences. Cell 25: 399-408, 1981.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05254-04 LB																								
PERIOD COVERED      October 1, 1981 to September 30, 1982																										
TITLE OF PROJECT (80 characters or less)  Gene Expression in <u>Xenopus laevis</u>																										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 40%;">I.B. Dawid</td> <td style="width: 30%;">Chief, Developmental Biochemistry Section</td> <td style="width: 10%;">LB NCI</td> </tr> <tr> <td>Other:</td> <td>B.K. Kay</td> <td>Guest Worker</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>Y.H. Chien</td> <td>Expert</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>T. Sargent</td> <td>Guest Worker</td> <td></td> </tr> <tr> <td></td> <td>B. Wood</td> <td>Lab. Worker</td> <td>LB NCI</td> </tr> <tr> <td></td> <td>U. Affolter</td> <td>Visiting Fellow</td> <td></td> </tr> </table>			PI:	I.B. Dawid	Chief, Developmental Biochemistry Section	LB NCI	Other:	B.K. Kay	Guest Worker	LB NCI		Y.H. Chien	Expert	LB NCI		T. Sargent	Guest Worker			B. Wood	Lab. Worker	LB NCI		U. Affolter	Visiting Fellow	
PI:	I.B. Dawid	Chief, Developmental Biochemistry Section	LB NCI																							
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COOPERATING UNITS (if any)  <div style="text-align: center;">None</div>																										
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TOTAL MANYEARS: <div style="text-align: center;">4.5</div>	PROFESSIONAL: <div style="text-align: center;">4</div>	OTHER: <div style="text-align: center;">0.5</div>																								
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SUMMARY OF WORK (200 words or less - underline keywords)  <p>The structure of a <u>repeated DNA</u> sequence that is expressed during development of <u>Xenopus laevis</u> has been studied. A conserved repeat unit of about 3.5 kb is flanked by clusters of tandemly repeated sequences with a 180 bp period. To study genes that are first expressed early in development we have prepared a <u>cDNA library</u> highly enriched for sequences expressed in <u>gastrula</u> but rare or absent in unfertilized eggs. We have isolated a cDNA clone derived from mRNA for the ubiquitous Ca-binding protein <u>calmodulin</u>; the sequence of this cDNA has been determined.</p>																										

## Project Description:

### Objectives:

The analysis of developmental expression of genes active in embryogenesis is the goal of this project. Special emphasis is given to those genes and their products which make their first appearance during development at the time of gastrulation. To compare the structure and regulation of a ubiquitously expressed gene with a tissue specific gene we are studying the genes for calmodulin and parvalbumin.

### Methods Employed:

cDNA made from Xenopus laevis gastrula mRNA was stripped of sequences that are also present in unfertilized eggs by hybridization to excess egg mRNA followed by hydroxylapatite chromatography. The resultant "gastrula-enriched" cDNA was cloned to generate an enriched cDNA library. A non-enriched cDNA library was also prepared from gastrula mRNA by standard technology.

Calmodulin sequences have been isolated by cloning of cDNA derived from mRNA of Xenopus testis and brain. Identification of the isolated DNA was achieved by sequencing according to the Maxam/Gilbert procedure.

A family of repeated DNA sequences that are expressed in Xenopus embryos was identified by the study of cDNA clones. cDNAs and portions of a corresponding genomic clone have been sequenced by the Sanger method after subcloning in M13 derivatives.

### Major Findings:

Among the nonmitochondrial transcripts in Xenopus that are moderately repeated in the genome we have looked for gene families encoding embryo-specific genes. Approximately 15% of the cDNA plasmids, from either the gastrula or tadpole cDNA libraries, contain sequences present at or above 500 copies per genome equivalent. Southern blots show that the majority of the repeat members appear to be interspersed in the genome. From a survey of cDNA clones we have found seven plasmids that together define a gene family of approximately 2,000 conserved units. Northern blots show two poly(A)+ RNA species, 6 and 10 kb, that are moderately abundant in the cytoplasm of neurula cells. These RNAs are less abundant, or absent, in earlier embryo stages. Genomic segments that hybridize to these cDNA plasmids have been recovered from a lambda library. These conserved repeated units are linked in the genome to tandem repeat clusters containing a 180 bp period. The nucleotide sequence has been determined for portions of these sequences from cDNA and genomic sources.

To study genes that are expressed very early in development a gastrula-enriched cDNA library has been generated as described under Methods. Ninety nonmitochondrial clones were selected from the enriched gastrula library and hybridized to egg and gastrula cDNA probes. Approximately half of the clones reacted much more strongly to the gastrula probe, a few showed no significant difference and the remainder failed to react detectably with either probe. This latter result was shown not to be due to small cDNA inserts in these clones. The

non-enriched gastrula library was also screened as above. Most (83/90) clones showed no significant difference, four increased from egg to gastrula and three decreased. Thus, an enriched gastrula cDNA library has been prepared.

We have isolated a cDNA clone of Xenopus calmodulin. Nucleotide sequence analysis indicates that it contains the entire coding region. The corresponding amino acid sequence is identical to that of bovine brain calmodulin except that at position 129 an Asn in bovine is replaced by an Asp in Xenopus. Northern analysis indicates that the major Xenopus calmodulin mRNA has a length of about 1.2 kb.

We have isolated parvalbumin from Xenopus and made antiserum to it. This will be used in efforts isolate the parvalbumin gene.

#### Significance to Cancer Research:

The aim of this project is a better understanding of developmental regulation of gene activity. This problem is of basic and general importance in understanding the behavior of normal and abnormal cells. During embryogenesis cell growth and differentiation is controlled precisely; an analysis of the molecular mechanisms of this control is the ultimate aim of this project.

#### Proposed Course of Research:

The repeated gene family described above, selected examples from the gastrula enriched library, and the calmodulin gene will be used to study expression in development. The abundance and size of mRNAs will be analyzed, the distribution of transcripts in different cells and cell compartments will be studied, and selected examples of genomic DNA sequences will be isolated from recombinant phage libraries. Some of these genes will be injected into Xenopus oocytes or embryos to study expression in a reconstructed system.

#### Publications:

Dworkin, M.B., Kay, B.K., Hershey, J.W.B. and Dawid, I.B.: Mitochondrial RNAs are abundant in the poly(A)<sup>+</sup> RNA population of early frog embryos. Develop. Biol. 86: 502-504, 1981.

Dawid, I.B. (rapporteur): Genomic change and morphologic evolution. In Evolution and Development, J.T. Bonner, ed., pp. 19-39. Springer Verlag, Berlin, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05258-03 LB
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PERIOD COVERED      October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Molecular Studies of Eukaryotic Gene Regulation

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	B.M. Paterson	Research Chemist	LB NCI
Other:	Z. Zehner	Guest Worker	LB NCI
	J. Eldridge	Biochemist	LB NCI
	A. Seiler-Tuyns	Fogarty Visiting Fellow	LB NCI
	R. Billeter	Fogarty Visiting Fellow	LB NCI
	R. Horlick	Graduate Student	LB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Biochemistry

SECTION

Developmental Biochemistry Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

2.5

PROFESSIONAL:

2.5

OTHER:

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☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☒ (c) NEITHER

☐ (a1) MINORS    ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Using cloned cDNA probes, we have isolated the genomic sequences for  $\alpha$  and  $\beta$  actin, vimentin, a major intermediate filament protein, and GAPDH (glyceraldehyde phosphate dehydrogenase). Preliminary characterization of the actin gene family suggests there are two different  $\alpha$  genes and one  $\beta$ -gene. DNA sequence studies around the 5' proximal intron in the 2  $\alpha$  gene isolates demonstrate complete coding sequence homology yet no homology within the intron. In vivo transcription of the vimentin gene, a single copy gene, yields two distinct mRNA transcripts by Northern analysis. Sequence studies of various vimentin cDNA clones demonstrate the 3' noncoding region of the gene contains 4 polyadenylation sites clustered in pairs approximately 300 base pairs apart. The in vivo mRNA transcripts differ by approximately 300 bp. The cDNA clone containing the poly A sites nearest the coding region hybridizes to both in vivo mRNA transcripts; the cDNA clone containing the more distal polyA sites hybridize only to the larger mRNA transcript. This suggests either polyadenylation cluster is used in vivo. The function of this heterogeneity is unclear at present. The GAP cDNA clone from chicken was used to isolate the homologous genes in yeast. Two of the three different yeast GAP genes were characterized with regard to transcription initiation and termination sites.



Project Description:

- (1) To prepare ds cDNA probes for the proteins of interest, and to utilize these probes to isolate the genomic sequences for structural studies. These studies primarily involve electron microscopic analysis and sequence analysis of all or part of the transcriptional unit for a given gene.
- (2) To define the transcriptional start sites, splice junctions and termination signals for the genes of interest. This information will be used in a comparative study of differentiation specific and "house keeping" sequences.
- (3) Some of the ds cDNA probes are to be used in chromosomal localization studies on mouse chromosomes. Several of the structural genes under investigation are highly conserved across species. It is of interest to know if isozymes are proximal or distal on the same chromosome, or on different chromosomes.
- (4) When the promotor regions for various genes have been identified, it is intended to analyze promotor function in one of the eukaryotic vector systems now available. We intend to: 1) see if a promotor for a differentiation specific gene, such as  $\alpha$  actin, can function when placed in an undifferentiated cell or if differentiation is required for function; 2) define the essential sequence elements for promotor function; 3) determine the role of the intron arrangement in the regulation of gene expression; 4) examine the role of polyadenylation in the regulation of gene expression.
- (5) Dr. Seiler-Tuyns who has just joined the laboratory, will examine the optimal parameters for the utilization of the eukaryotic vector systems now available. This will be done with various constructs of the histone H4 gene from mouse which she has characterized in her doctoral studies. .

Methods Employed

Specific ds cDNA probes are being prepared from A<sup>+</sup> mRNA fractionated on preparative methylmercury gels. Fractions enriched for a particular mRNA, as judged by analysis in a cell-free protein synthesizing system, are cloned using standard methods. Clones are identified by positive selection.

The probes are used to screen the charon 4A library of the chicken genome with standard procedures, or with the supressor miniplasmid method of Maniatis. The distribution of the coding information and its polarity within a given isolate is determined by hybridization with kinased RNA and short cDNA. The 5' proximal and 3' proximal fragments in the appropriate restriction digest are sequenced to clearly determine the end points of the transcriptional unit for each gene. Electron microscopic studies give the information on the intron-exon pattern and the relatedness of the various isolates for a given gene. Restriction fragments containing the promoter regions will be subcloned into one of the eukaryotic vector systems for further analysis.



Major Findings:

Actin. We have characterized, in part, 2 distinctly different  $\alpha$ -actin (skeletal muscle actin) genomic clones by restriction enzyme analysis and nucleotide sequence. Sequence studies at the intron/exon junction for each gene at amino acid position 41/42 reveal complete homology within each exon yet the intron sequences demonstrate no obvious homology. Efforts to obtain the promotor sequence for each gene are continuing in order to facilitate more detailed comparisons both structurally and functionally.

Our initial  $\beta$ -actin genomic clone did not contain the complete gene so the library was rescreened and isolates containing the entire gene have now been prepared as judged by hybridization studies with 5' and 3' probes. Restriction maps for these new isolates have been completed and nucleotide sequence studies of the 5' region of the gene are under way. All the  $\beta$ -gene isolates prepared to date appear to overlap, unlike the  $\alpha$ -actin isolates.

Preliminary copy number studies indicate there are 2  $\alpha$  actin genes and a single  $\beta$  actin gene. Both  $\alpha$  sequences appear to be in a single 20 kb R1 fragment. The  $\beta$  gene is contained in a 24 kb R1 fragment.

Vimentin. Detailed analysis of the genomic clones for the intermediate filament protein, vimentin, have continued. Copy number experiments indicate there is one copy of the gene per haploid genome; comparisons of the restriction digests of genomic DNA and the cloned gene yield identical patterns, and, reconstruction experiments indicate there is a single gene. Analysis of either total or polyA<sup>+</sup> mRNA extracted from muscle or other tissue and sized on methylmercury hydroxide gels (Northern analysis) yields two distinct mRNAs differing in molecular weight by approximately 300 nucleotides. Both mRNAs are functional in vitro and give the expected polypeptide by 2D gel analysis. Detailed nucleotide sequence analysis of the 3' nontranslatd portion of the gene utilizing various cDNA clones representing the 3' end of the gene indicates there are 4 polyadenylation sites clustered in two groups, 300 nucleotides apart. Furthermore, the cDNA clone containing the pair of polyadenylation sites nearest the 3' end of the coding region hybridizes to both in vivo mRNA transcripts. The cDNA clone containing the more distal polyadenylation sites hybridizes exclusively to the larger mRNA transcript. This suggests either polyadenylation cluster is used in vivo and provides a mechanism for the transcription of 2 different mRNAs from the same gene. The function of this mRNA heterogeneity is unclear at present. Vimentin does not appear related to the keratins at the nucleotide level.

Glyceraldehyde phosphate dehydrogenase. Using the GAP cDNA clone prepared from embryonic chick muscle mRNA and taking advantage of sequence homology, we have isolated two of the three different GAP genes in yeast. The yeast genes have been characterized with regard to their transcription initiation and termination sites, size, and intron/exon pattern. The genes contain no introns. Transcription initiates and terminates within sequences that are repeated on each end of the gene. Furthermore, the repeats are unique for each gene. The function of these repeats is not clear but is under investigation. Comparative studies of the yeast and chicken gene are under way.

Projected Course of Research:

We intend to analyze the organization, structure, and regulation of constitutive and differentiation specific genes.

Publications:

Alvino, C.G., Tassi, V., Paterson, B.M. and DiLauro, R.: In vitro synthesis of 30000 M<sub>r</sub> rat thyroglobulin subunit. FEBS Lett. 137: 307-313, 1982.

Miller, J.S., Roberts, B.E. and Paterson, B.M.: Determination of the organization and identity of eukaryotic genes utilizing cell-free translation systems. In: Setlow, J.K. and Hollaender, A. (eds.): Genetic Engineering, Vol. 4, Plenum, New York, in press.

Miller, J.S., Paterson, B.M., Riccardi, R.P., Cohen, L. and Roberts, B.E.: Methods utilizing cell-free protein synthesizing systems for the identification of recombinant DNA molecules. In: Grossman, L. and Moldave, K. (eds.): Methods in Enzymology, Academic Press, New York, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05261-03
PERIOD COVERED September 30, 1981 to October 1, 1982		
TITLE OF PROJECT (80 characters or less)  Manifestations of Human Cancer		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Cecil Fox Senior Scientist LB NCI		
COOPERATING UNITS (if any) Department of Biochemistry, Lunds Universitet, Lund, Sweden; Departments of Surgery and Oncology, Walter Reed Army Medical Center; ALERT Leprosy Hospital, Addis Ababa Ethiopia; SSDZ Department of Anatomical Pathology Delft, Holland; Department of Histology, Karolinska Inst., Stockholm		
LAB/BRANCH Laboratory of Biochemistry		
SECTION Office of the Chief		
INSTITUTE AND LOCATION DCBD, NCI, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 1	PROFESSIONAL: 1	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The goals of this program are to use <u>biological characteristics of tumor cells</u> as a means for improving <u>grading of human cancer</u> through use of more objective parameters. The object of <u>grading tumors</u> is to estimate the potential of the tumor for growth, <u>invasion</u> , and <u>metastasis</u> which are important factors to clinicians in planning therapy. <u>Tumor cell attachment</u> to new substrates is being compared to normal cell attachment using reflection contrast microscopy. A major characteristic of cancer cells, release from <u>density dependent growth control</u> , is also being studied as is the role of <u>lamellar cytoplasm</u> in tumor cell growth. A long-term study of the pathology and dynamics of <u>infections in immune compromised patients</u> is in progress. A preliminary investigation of the <u>histiogenesis of lepromatous</u> and <u>histoid leprosy</u> as a model of mesenchymal dysplasia has been started. One project characterizes human cancer cell <u>clonogenic populations</u> in archival tissues and with <u>needle aspiration cytology</u> . A study of endometrial epithelium and its growth dynamics in relation to endometrial hyperplasia is now underway, and a separate project on the dynamics of cellular response to <u>cytostatic agents</u> in head and neck cancer is in progress.		

## Project Description

Objective: The grading of cancers is an estimate of the biological character of a tumor cell population and is usually done by subjective appraisal. This project is designed to provide the pathologist and the clinician with objective signs and data bases that may be included in the information used in deciding the course of treatment of human cancers. The project is a broad one that seeks to identify characteristics of human carcinomas that may be used for interpretation of the biological state of cancerous or precancerous cells.

Methods Employed: The methods employed will be described under each study heading.

### Study 1. Cell attachment mechanisms and the formation of cell contacts with substrates

The attachment of cells to substrates may be involved in the process of metastasis and could perhaps be used for characterizing cellular populations of cancers. The mechanics of cell attachment to glass surfaces using reflection contrast microscopy continue, and as more cells become available, time-lapse cinemicrographs will be made of the process so that the dynamics of this differentiation process in epithelial cells may be identified.

### Study 2. Cytological characterization of clonogenic and non-clonogenic cells in human head and neck tumors

The premise of clonogenic assays is: that only a small number ( $1 \times 10^{-6,-8}$ ) of cells from a human cancer are growing, that these cells contribute to the patient's cancer, and that these cells eventually form fatal metastases. This ongoing project attempts to test this hypothesis by establishing the number of cells in S-phase in a given tumor and to determine the amounts of DNA in the mitotically active cells. Further, the project will follow which tumor cell populations, classified by morphometry and DNA content, are most susceptible to chemotherapy.

### Study 3. Metaplasia in epithelium

The process of metaplasia in epithelial surfaces is often believed to be a first step in the carcinogenic process. It is possible to introduce metaplasia in vitro. This study concerns the cellular events of metaplasia as determined by light and electron microscopy and will try to establish which cell populations proliferate to metaplasia.

### Study 4. Quantitative parameters useful in differentiating endometrial hyperplasia from endometrial carcinoma and CIS.

Exploratory studies have shown that endometrial tissues that subjectively are of similar structure could either be only endometrial hyperplasia or endometrial carcinoma. Known cases of endometrial carcinoma will have glandular epithelial cells that are aneuploid while those from hyperplasias will rarely exceed 4n amounts of DNA. This added factor may be of use to pathologists in decision-making and while not diagnostic, provides one more piece of information in the



logic of diagnosis. With known archival specimens it will be possible to investigate other cellular parameters such as nuclear density of stromal cells, or of geometric relationships between cell types.

#### Study 5. The dynamics of infectious agents in immune compromised patients

Cancer patients are frequently treated with immune suppressive steroids, with chemotherapeutic agents, or often, with both. The diversity and virulence of these infections is well-demonstrated by the cases contributed to the AFIP which serves as a repository for unusual agents from the entire world. We are conducting a retrospective study of the range and diversity of adventitious agents in immunosuppressed patients that will improve the rapidity of pathologic judgment of diatheses.

#### Study 6. The role of lamellar cytoplasm in neoplastic transformation

A study continues on lamellar cytoplasm in growth regulation of cells. Cells growing on surfaces continue to grow when they can spread their cytoplasm maximally. When cell crowding becomes great enough, cells cease to divide and may go over to the  $G_0$  phase of the cell cycle. While not entirely similar to human epithelium, rabbit renal epithelium may be dissected in the form of renal tubules and a specific portion of the tubule cultured on collagen membranes. We are currently studying the growth of such monolayers in relation to development of renal function by the epithelial cells, and the effect of microfilament reorganization on lamellar cytoplasm.

#### Study 7. Lepromatous and histoid leprosy as models of mesothelial dysplasia.

Long-term hyperplasia of mesothelial cells in humans is a relatively uncommon event. One example is the lesions of histoid and lepromatous leprosy. A morphometric study of hyperplasia of such cells is being made in drug resistant strains of leprosy.

#### Significance:

Cancer in humans may be part of the human estate: that is, those events that lead to cancer might also lead to the irreversible differentiation of the cells of human epithelium. If this is true, that cancer is the result of some number of transpositions or even eradications in the genome, then the most immediate benefit to the cancer patient would be research to improve his chances with existing treatment modalities. This program is intended to investigate such a possibility and further, to investigate some of the cell biological characteristics of epithelial cancer cell populations in humans whenever possible. The research projects in this program have the modest but important goal of trying to obtain methods for improving decision-making about tumor populations.

#### Proposed Course of Research

Because of the limited resources available and the large number of projects described above, progress will of necessity be piecemeal. Studies 2 and 3 are essentially new studies that have only begun, but will continue as resources allow.



Publications

Boone, M.E. and Fox, C.H.: Simultaneous condyloma acuminatum and dysplasia of the uterine cervix. Acta Cytologica 25: 393-399, 1981.

Fox, C.H. and Piekarski, L.: Biological manifestations of cancerogenesis. Postepy Microbiologii 20: 15-26, 1981.

Fox, C.H.: Why do cells stick? Karyogram 8: 23, 1982.

Rabinowitz, M., Bahr, H.J., Foster, W., Kurman, R.J., and Fox, C.H.: Predicting the outcome of endometrial hyperplasia: analysis of cell nuclear features using a linear discriminant function. Human Pathology, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05262-02 LB																											
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>																													
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Eukaryotic Gene Regulation and Gene Transfer</p>																													
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: D.H. Hamer</td> <td style="width: 33%;">Senior Staff Fellow</td> <td style="width: 33%;">LB NCI</td> </tr> <tr> <td colspan="3">Others:</td> </tr> <tr> <td>G.N. Pavlakis</td> <td>Fogarty Fellow</td> <td>LB NCI</td> </tr> <tr> <td>A.D. Carter</td> <td>Guest Worker</td> <td>American Cancer Society</td> </tr> <tr> <td>M.F. Jubier</td> <td>Guest Worker</td> <td>CNRS</td> </tr> <tr> <td>B. Felber</td> <td>Fogarty Fellow</td> <td>LB NCI</td> </tr> <tr> <td>C.S. Schmidt</td> <td>Graduate Student</td> <td>FAES</td> </tr> <tr> <td>A. Kumar</td> <td>Guest Worker</td> <td>George Washington University</td> </tr> <tr> <td>M.J. Walling</td> <td>Chemist</td> <td>LB NCI</td> </tr> </table>			PI: D.H. Hamer	Senior Staff Fellow	LB NCI	Others:			G.N. Pavlakis	Fogarty Fellow	LB NCI	A.D. Carter	Guest Worker	American Cancer Society	M.F. Jubier	Guest Worker	CNRS	B. Felber	Fogarty Fellow	LB NCI	C.S. Schmidt	Graduate Student	FAES	A. Kumar	Guest Worker	George Washington University	M.J. Walling	Chemist	LB NCI
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C.S. Schmidt	Graduate Student	FAES																											
A. Kumar	Guest Worker	George Washington University																											
M.J. Walling	Chemist	LB NCI																											
COOPERATING UNITS (if any)  <p style="text-align: center;">None</p>																													
LAB/BRANCH  <p style="text-align: center;">Laboratory of Biochemistry</p>																													
SECTION  <p style="text-align: center;">Cellular Regulation Section</p>																													
INSTITUTE AND LOCATION  <p style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</p>																													
TOTAL MANYEARS: <p style="text-align: center;">6</p>	PROFESSIONAL: <p style="text-align: center;">5</p>	OTHER: <p style="text-align: center;">1</p>																											
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																							
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																													
SUMMARY OF WORK (200 words or less - underline keywords)  <p>           We have used <u>recombinant DNA techniques</u> to construct <u>DNA tumor virus hybrids</u> carrying various <u>chromosomal eukaryotic genes</u>. These recombinant molecules have been introduced into cultured mammalian cells by infection, transfection or transformation. Such experiments allow us to identify <u>regulatory sequences</u>, such as those required for the heavy metal-inducible transcription of the <u>mouse metallothionein-I gene</u>; to overproduce useful gene products, such as <u>human growth hormone</u>; and to determine the effects of naturally occurring mutations, such as a deletion that causes a <u>human thalassemia</u>.         </p>																													

## Objectives

We wish to understand the regulated expression of animal cell genes.

## Methods Employed

Our general strategy is to use SV40 or other DNA tumor virus vectors to introduce the gene of interest, and mutants derived from it, into cultured cells where their expression can be studied in detail.

## Major Findings

### 1) Regulation of Metallothionein Expression

Cadmium and other heavy metals induce metallothionein synthesis in mammalian cells. We cloned a mouse metallothionein-I gene from a cadmium-resistant line of Ltk<sup>-</sup> cells and inserted it into viral and plasmid SV40 vectors. These recombinant molecules were introduced into cultured monkey cells (either by infection or transfection) and the expression of the foreign metallothionein gene was analyzed, at the RNA level, in the presence and absence of cadmium. The results show that the cloned gene retains its cadmium inducibility and that the regulation of this gene occurs primarily at the transcriptional level.

The DNA sequences responsible for this form of regulation are being investigated by in vitro mutagenesis. A large collection of deletion mutants, covering both the 5' flanking and intergenic sequences, have been constructed and inserted into plasmid SV40 vectors. In addition, we have established that the transcriptional activity of such mutants can be conveniently assayed using the *E. coli* galactokinase read-through assay developed in Dr. Martin Rosenberg's laboratory.

We have also initiated a project to isolate and characterize primate metallothionein genes. Two cDNA clones were obtained from cadmium-resistant monkey kidney cells and completely sequenced. These clones encode two distinct metallothioneins as shown by their high cysteine content, complete lack of aromatic amino acids and homology to the known amino acid sequences of metallothioneins from other species. Further, these clones have been used as probes to isolate the human genomic clones in phage  $\lambda$ . These should be useful for identifying evolutionarily conserved sequences important for heavy metal regulation and for analyzing the molecular mechanisms of human disorders in heavy metal metabolism.

### 2) Protein Production

Two different human growth hormone (hGH) genes were inserted into SV40 vectors. Monkey kidney cells infected with these recombinants synthesize, process, and secrete hGH. The product of gene 1, which has coding sequences identical to those of a cloned hGH complementary DNA, is indistinguishable from pituitary hGH by several criteria. The product of gene 2, which is predicted to encode a variant protein, is less immunoreactive than pituitary

hGH but binds efficiently to hGH cell surface receptors. These results show that gene 2 has the potential to be expressed into a previously unidentified form of hGH. They also demonstrate that it is possible to produce a mature hormone by gene transfer in eukaryotic cells and indicate the utility of the simian virus 40-monkey cell system for producing and characterizing secreted animal cell proteins.

More recently we have fused the hGH structural sequences to the mouse metallothionein-I gene promoter and introduced the hybrid gene into mouse cells on a bovine papilloma virus vector. The stably transformed cells produce large quantities of hGH and the synthesis of this protein is inducible by cadmium. This system should be useful both for overproducing desirable gene products and for testing the physiological roles of proteins with unknown functions.

### 3) RNA Splicing

The  $\alpha 2$ -globin gene from an  $\alpha$ -thalassemia patient contains a pentanucleotide deletion in the first intron immediately adjacent to the first exon. We have tested the functional consequences of this mutation by introducing the thalassemic gene, along with its normal counterpart as a control, into cultured monkey cells on SV40 plasmid vectors. Both genes are expressed, at similar levels, into globin RNA with the correct 5' and 3' ends. However, while most of the normal transcripts are appropriately processed, the thalassemic transcripts are abnormally spliced from a 5' donor site in the middle of the first exon to the normal 3' acceptor site. This results in the synthesis of a truncated RNA that is incapable of encoding a normal globin polypeptide. The alternative donor, which is also used at a low level in monkey cells transfected with the normal gene, shows strong homology to the consensus donor sequence characteristic of many eukaryotic splice junctions. No unspliced or partially spliced thalassemic RNA was detected, indicating that recognition of this site is efficient and does not block removal of the second intron. Alternatively spliced RNA was also found in bone marrow RNA from the  $\alpha$ -thalassemia patient although not in marrow from a normal individual or a  $\beta^+$ -thalassemia patient. These results show that the thalassemic phenotype of the patient results from abnormal RNA splicing due to the deletion of the first splice donor signal.

### 4) tRNA Processing

In collaboration with Dr. Michael Zasloff we constructed an SV40 recombinant carrying a mutant human tRNA<sup>met</sup> gene. The mutant gene has a G  $\rightarrow$  T transversion of position 56 and was previously demonstrated to block normal processing of the pre-tRNA in an in vitro system. Monkey cells infected with this recombinant efficiently transcribed the variant gene; however, the precursor RNA was not processed to a mature tRNA species.

Publications

Hamer, D.H.: The introduction of normal and mutant globin genes into mammalian cells using SV40 vectors. In: Molecular Basis of Mutant Hemoglobin Dysfunction. P. Sigler, ed., Elsevier, New York, pp. 97-102, 1981.

Pavlakakis, G.N., Hizuka, N., Gorden, P., Seeburg, P. and Hamer, D.: Expression of two human growth hormone genes in monkey cells by cloning in simian virus 40. Proc. Natl. Acad. Sci. USA, 78: 7398-7402, 1981.

Zasloff, M., Santos, T. and Hamer, D.H.: tRNA precursor transcribed from a mature human gene inserted into a SV40 vector is processed incorrectly. Nature 295: 533-535, 1982.

Hamer, D.H. and Walling, M.J.: Regulation in vivo of a cloned mammalian gene: Cadmium induces the transcription of a mouse metallothionein gene in SV40 vectors. J. Mol. Appl. Genet., in press 1982.

Felber, B.K., Orkin, S.H. and Hamer, D.H.: Abnormal RNA splicing causes one form of  $\alpha$ -thalassemia. Cell, in press 1982.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)		U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 05263-01 LB	
PERIOD COVERED October 1, 1981 to September 30, 1982					
TITLE OF PROJECT (80 characters or less) Eukaryotic Chromatin Structure and Gene Regulation					
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT					
PI: C. Wu		Visiting Associate		LB NCI	
Other: T. Paisley		Biologist		LB NCI	
COOPERATING UNITS (if any)  None					
LAB/BRANCH Laboratory of Biochemistry					
SECTION Developmental Biochemistry Section					
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205					
TOTAL MANYEARS: 1.4		PROFESSIONAL: 1.4		OTHER: 0	
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS					
SUMMARY OF WORK (200 words or less - underline keywords)  <p>The orderly compaction of DNA in <u>nucleosomes</u> along the <u>chromatin</u> fiber is punctuated by highly nuclease-sensitive sites. We found such <u>hypersensitive</u> sites in cellular chromatin by mild cleavage of chromatin in isolated nuclei with <u>DNaseI</u>, and Southern analysis of the partially cut DNA using <u>cloned hybridization</u> probes. We showed that the DNase I hypersensitivity is located at the 5' terminus of several heat-inducible (heat shock) genes in <u>Drosophila</u>, and is present during and before induction. Currently we are trying to probe the fine structure of the DNase I hypersensitive site using DNase I and restriction endonuclease digestion of chromatin, and using high resolution Southern blots to analyze the cleavage patterns. We suggest that preferentially accessible sites in chromatin such as these define a state of potential activity for eukaryotic genes, and may function as entry sites to the DNA sequence for RNA polymerase and control factors.</p>					

## Project Description:

### Objectives:

A knowledge of gene regulation is fundamental to an understanding of eukaryotic development and differentiation. We study gene regulation by probing the state of DNA in its native state, as chromatin. We have already shown that for several genes, the 5'-terminal and flanking sequences, a region acknowledged to be important for control, is uniquely accessible in chromatin to a nucleolytic probe, DNase I. We aim to further characterize the DNase I hypersensitive sites in chromatin.

### Methods Employed:

We are concentrating on studying the chromatin structure of the heat-inducible genes in *Drosophila* which encode the 70K, 83K and 68K heat shock proteins (hsp 70, hsp 83, hsp 68). Cloned DNA representing these genes have been generously provided by members of Dr. M. Meselson's laboratory at Harvard University. The fine structure of the DNase I hypersensitive sites in the chromatin around these genes are being mapped by the indirect end-labeling method which we developed previously. We are now separating the partially cleaved DNA segments on 50 cm agarose gels, which can give a length determination precise to 5-10 bp in the 1 to 2 kilobase pair range.

### Major Findings:

Using this high resolution mapping, we are able to relate the fine structure of the DNase I hypersensitive site at the 5' end of the hsp 70 gene to the published DNA sequence. Our results show that the 5' upstream flank of the gene is hypersensitive from -38 to -215 bp; the 5' downstream flank is hypersensitive from -8 to +100 bp. Most interestingly, the 30 bp region in between, from position -8 to -38, which contains the TATAAAT sequence, is relatively protected from cleavage. There is also a point of greatest hypersensitivity at position -93.

### Significance to Cancer Research:

Our work contributes to a broad effort in the study of gene regulation during normal development and differentiation, and will serve as a basis for the study of aberrant cellular functions which result in neoplasia.

### Proposed Course of Research:

We plan on doing a similar study on hsp 83 and hsp 68, and other *Drosophila* genes, to look for similarities and differences.

We have begun experiments using restriction enzymes as structural probes of chromatin to further complement our DNase I studies. Our early results suggest a parallel response with restriction enzymes, i.e., chromatin regions hypersensitive to DNase I are also hypersensitive to restriction enzymes whose sites are located therein.

Publications:

Wu, C.: An exposed chromatin structure at the 5' end of eukaryotic genes. In: O'Malley, B. and Fox, C.F. (eds): ICN-UCLA Symposium on Molecular and Cellular Biology, Vol. XXVI, New York, Academic Press, in press.

Wu, C.: Chromatin structure of Drosophila heat shock genes. In: Ashburner, A., Tissieres, A. and Schlesinger, M, (eds): Heat Shock Induction of Proteins. Cold Spring Harbor Publication, New York, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 05264-01 LB
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Characterization of a Mouse Repetitive Gene Family</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	K.K. Lueders	Chemist
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		LB NCI
COOPERATING UNITS (if any)  <p style="text-align: center;">S. Segal, Nucleic Acid Enzymology Section, LB/DCBD          B. Paterson, Developmental Biochemistry Section, LB/DCBD</p>		
LAB/BRANCH  <p style="text-align: center;">Laboratory of Biochemistry</p>		
SECTION  <p style="text-align: center;">Biosynthesis Section</p>		
INSTITUTE AND LOCATION  <p style="text-align: center;">DCBD, NCI, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS:  <p style="text-align: center;">0.5</p>	PROFESSIONAL:  <p style="text-align: center;">0.5</p>	OTHER:  <p style="text-align: center;">0</p>
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SUMMARY OF WORK (200 words or less - underline keywords)		
<p>           We have identified and characterized a family of interspersed repetitive sequences from the mouse genome. Individual members of the family (designated MIR-1) have been isolated from mouse genomic libraries. The repeats are about 400 base pairs long and amount to 1-2% of mouse genomic DNA. The sequences represent a group of homologous but non-identical units, and individual members of the family show considerable divergence from one another. The spatial relationships between members of the family and a number of other identified mouse sequences including structural genes have been determined; these sequences are found on the 5' as well as 3' sides of genes at distances ranging from less than 1 to 5 kilobases. The sequences are present in the DNA of all species of <u>Mus</u>. Related sequences are present in the rat genome at a repetition frequency similar to that in the mouse genome.         </p>		

Structural studies: We have used restriction analysis and heteroduplex formation to define a repetitive family of sequences in the mouse genome. The size of the sequence element is about 400 base pairs. We refer to this element as MIR-1 (mouse interspersed repeat-1). Twenty-seven percent of the clones in a mouse genomic library reacted with MIR-1. The intensity of the reactions was relatively uniform, suggesting the sequences are interspersed in the genome. Individual members of the family are considerably diverged ( $\Delta T_m = 12^\circ$ ) from one another as determined from melting curves of hybrids formed with different cloned isolates of MIR-1 family members and genomic DNA from M. musculus.

Evolutionary studies: Genomic DNAs from seven species of Mus all contained MIR-1 sequences. The  $T_m$  of hybrids formed with M. musculus and a distantly related Asian mouse species (M. cervicolor) DNAs differed by only  $2.5^\circ\text{C}$ , indicating the MIR-1 sequences in M. cervicolor are as divergent from those in M. musculus as are individual members of the family within M. musculus. Mouse MIR-1 sequences reacted with the same percentage of clones in a rat genomic library as in a mouse genomic library, indicating that the reiteration frequency of MIR-1 sequences in the mouse and rat genomes appears to be similar, and the sequences in rat also seem to be dispersed. Reactions were also seen with Syrian hamster and African green monkey DNAs, but these were much weaker than those with rat and mouse DNAs.

Relationship between MIR-1 and other identified mouse sequences: We have tested over 100 recombinants selected in other laboratories because they contained identifiable mouse genes for MIR-1 sequences. MIR-1 sequences occur on both the 5' and 3' sides of genes at distances of less than 1 Kb to over 5 Kb. Among genes which had MIR-1 sequences in their proximity were: several  $\beta$ -globin genes, an  $\alpha$ -globin related gene, K light chain variable region genes, partial AKV genes, H2 genes,  $\alpha$ -amylase gene, and several intracisternal A-particle genes.

Functional studies: One member of the MIR-1 family selected by virtue of its ability to promote transcription of a bacterial gene is being studied in collaboration with Dr. S. Segal for promoter activity in mammalian cells using transfection experiments with the cloned DNA.

Future plans: Studies on promotion of transcription by MIR-1 sequences in mammalian cells will continue. We will also determine if the sequences can be detected in the RNA of mouse cells, particularly in nuclear RNA. Sequencing of MIR-1 will be carried out by Dr. B. Paterson to determine the nature of the element, and to define its relationship to other repetitive elements in the mouse genome.

Significance: The role of DNA rearrangements as a means of changing the levels of expression of cellular genes is becoming a subject of great interest. One type of sequence shown to play such a role in activating the expression of cellular oncogenes is retroviral long terminal repeats. Other classes of repetitive sequences with promoter activity might have similar effects and, therefore, it is of interest to identify and characterize such sequences.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08701-08 LB																											
PERIOD COVERED October 1, 1981 to September 30, 1982																													
TITLE OF PROJECT (80 characters or less)  Regulation of Cellular and Viral Gene Expression																													
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<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: M. Rosenberg</td> <td style="width: 33%;">Research Chemist</td> <td style="width: 33%;">LB NCI</td> </tr> <tr> <td colspan="3"> </td> </tr> <tr> <td>OTHERS: Y. Ho</td> <td>Visiting Fellow</td> <td>LB NCI</td> </tr> <tr> <td>M. Reff</td> <td>Sr. Staff Fellow</td> <td>LB NCI</td> </tr> <tr> <td>A. Shatzman</td> <td>IPA</td> <td>Georgetown University</td> </tr> <tr> <td>D. Sobieski</td> <td>Chemist</td> <td>LB NCI</td> </tr> <tr> <td>G. Duester</td> <td>Guest Researcher</td> <td>LB NCI</td> </tr> <tr> <td>H. Johansen</td> <td>Visiting Fellow</td> <td>LB NCI</td> </tr> <tr> <td>G. Ramos</td> <td>Lab Worker</td> <td>LB NCI</td> </tr> </table>			PI: M. Rosenberg	Research Chemist	LB NCI				OTHERS: Y. Ho	Visiting Fellow	LB NCI	M. Reff	Sr. Staff Fellow	LB NCI	A. Shatzman	IPA	Georgetown University	D. Sobieski	Chemist	LB NCI	G. Duester	Guest Researcher	LB NCI	H. Johansen	Visiting Fellow	LB NCI	G. Ramos	Lab Worker	LB NCI
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SUMMARY OF WORK (200 words or less - underline keywords)    We have been investigating <u>nucleic acid structure</u> involved in the control of <u>transcription initiation and termination</u> as well as in <u>translational expression</u> of various RNAs. Using recombinant DNA techniques we have been able to examine the relationship between regulatory function and DNA sequence in a variety of cellular and viral operon control regions. The characterization of mutants which affect both transcriptional and translational expression in these regions has afforded information on precise molecular events which lead to control element function. We have used a variety of defined prokaryotic genes to examine the requirements for translation in both eukaryotic cells and cell-free systems. Recombinant DNA techniques have been used to develop plasmid, phage and bacterial vector systems which allow the isolation, characterization, and comparison of prokaryotic transcriptional regulatory signals. This system has been adapted to allow efficient expression of a variety of prokaryotic and eukaryotic gene products in <u>E. coli</u> . Efficient expression of the <u>E. coli galactokinase</u> gene attached to a <u>mammalian virus vector</u> has been obtained within mammalian cells. This vector has allowed us to study transcriptional regulatory elements which function in eukaryotic cells.																													

Project Description:Objectives:

1. To examine the relationship between function and DNA structure involved in the control of transcription initiation and termination of RNA synthesis, as well as in the translational expression of these mRNAs in both prokaryotic and eukaryotic systems.
2. To study the role of transcription termination and RNA processing in the control of phage and bacterial gene expression.
3. To investigate the ability of defined bacterial and phage mRNAs to be efficiently translated in eukaryotic cells and cell-free systems.
4. To develop new techniques which can be applied to the above studies and to the general examination of nucleic acid structure in other biological systems.

Methods Employed:

Purification of specific RNA transcripts synthesized either in vivo or in vitro; nucleic acid sequence analysis of both RNA and DNA; primed extension of RNA transcription products with deoxyribonucleotides; isolation of protein factors involved either in the processes of transcriptional termination or post-transcriptional modification of RNA; isolation of purified DNA restriction enzymes and their employment in obtaining specific purified fragments of both viral and cellular DNAs; recombinant DNA techniques.

Major Findings:

1. The nucleic acid structure of the 3'-terminal regions of several RNA transcripts synthesized in vitro and in vivo from both phage and bacterial systems are being examined. These RNAs represent both independently terminated transcription products as well as transcripts requiring the protein factor, Rho, for their termination. Sequence analysis at the 3'-ends of some of these products indicated:
  - a) that all of the transcripts examined possess similar degrees of 3'-terminal sequence heterogeneity which consisted predominantly of the addition of 1 to 5 adenylate residues to the 3'-terminus of the transcript and
  - b) that rho factor enhanced termination results in a definite structural change in the nucleotide sequence with which an RNA molecule can terminate.

In addition, we have developed a new methodology for examining the nucleic acid structure in the untranscribed portion of the DNA immediately adjacent to the 3'-terminus of an RNA transcript. This technique has been applied to the determination of sequence information throughout the entire termination region of these transcription products. Little structural homology is apparent in the template DNA beyond the 3'-ends of these transcripts. The lack of homology suggests that this region might not be important to the termination process. Thus, the majority of the encoded termination signal is transcribed into RNA.

We determined the DNA sequence surrounding and examined termination at a totally rho dependent site (t<sub>R1</sub>) in bacteriophage  $\lambda$ . The DNA composition at t<sub>R1</sub> is 75% AT base pairs and termination occurs heterogeneously over four adjacent base pairs. No GC rich or U rich sequence is found at the 3'OH end of the RNA. This sequence is quite different from other transcripts that terminate independently of Rho. However, there is a stable base-paired stem and loop structure near the end of this RNA.

In the absence of Rho factor with normal transcription conditions (i.e. 37° and high triphosphate levels), RNA polymerase undergoes a substantial pause at this site. A mutation, cnc, that interferes with base pairing in the stem and loop, has two effects on transcription: RNA polymerase does not pause in the absence of Rho and transcription does not terminate in the presence of Rho. This suggests that the stem and loop induces RNA polymerase to pause, and that a paused polymerase is important for Rho action.

We have now completed a far more extensive characterization of the entire intercistronic region in which this termination site is positioned. A number of related transcriptional and translational regulatory elements have been defined in this region. More mutations affecting terminator function have been characterized. In addition, we have defined a site (NutR) located immediately preceding the terminator which is apparently involved in anti-termination function. We have shown that the viral function, N, acts in some way to prevent termination (i.e. anti-terminate) at the t<sub>R1</sub> site thereby allowing for the quantitative and temporal control of the transcription through this intercistronic region (i.e. transcription attenuation). Our data indicate that the NutR site comprises an ~17 nucleotide sequence which displays a hyphenated two-fold rotational symmetry. This site is in some way involved in N protein recognition prior to the action of N at the various downstream termination sites.

2. The galactose operon of E. coli is subject to both negative control by gal repressor and positive control by cAMP and its receptor protein (CRP). We have sought to define the nucleotide sequence of the promoter-operator sites responsible for gal regulation. Analysis of transcripts of restriction fragments combined with direct DNA sequencing enabled us to derive the sequence for the gal regulatory region. Cleavage by restriction endonuclease Hinf abolishes cAMP-CRP dependent but not CRP independent transcription. Formation of a cAMP-CRP dependent preinitiation complex prevents cleavage by Hinf. Thus the region upstream of the Hinf site is important for cAMP-CRP stimulation of transcription. This region contains sequence similarities with the CRP recognition site of lac. One gal operator constitutive mutant is resistant to Hinf cleavage suggesting the operator may include at least part of the Hinf site. Further analyses are required to confirm this possibility.

More recently we have demonstrated in vitro the existence on the plasmid pBR322 of a promoter signal that is strictly dependent on cAMP and its receptor protein CRP. Transcription initiates with pppG at nucleotide 2270 and proceeds counterclockwise on the standard pBR322 map. DNase protection studies show that CRP selectively binds to the -35 region of the promoter. This region

exhibits strong structural homologies to the binding sites of other CRP-dependent promoters.

In addition, we have been using an RNA-dependent *E. coli* S30 translation system to examine the translational efficiencies of in vitro synthesized mRNAs. Because the products of both the transcription and translation reactions can be separately quantitated, the amount of protein produced per mole of a specific mRNA can be determined. Using this method, we have compared the relative translational efficiencies of two different mRNA transcripts of the *E. coli* galactose operon: the CRP-cAMP dependent mRNA (P1) and the CRP-cAMP independent mRNA (P2). Our results show that the P2 mRNA translates epimerase, the 5' proximal gene product of the gal operon, 4x more efficiently than does the P1 transcript, while the 5' distal gene product, kinase, is translated with equal efficiency. Since the P2 transcript differs from the P1 transcript only by the addition of 5 nucleotides at the 5' terminus, and these nucleotides are outside of the ribosome binding region for epimerase, the selective difference in the translational efficiency of epimerase may be mediated by RNA conformation. It is known that in cells deficient in cAMP, the ratio of epimerase to kinase is about 4x higher than in cells containing cAMP - a phenomenon called discoordinate expression. Moreover, cells deficient in cAMP are thought to produce only gal P2 mRNA, whereas cells with cAMP produce only P1 mRNA. Thus, discoordinate expression is explained by our observation that the P2 transcript produces 4x more epimerase, but a similar amount of kinase, then does the P1 transcript.

These studies have been extended to examine the apparent coupled expression of the two distal genes of the gal operon, transferase (galT) and kinase (galK). Using recombinant DNA techniques we have introduced a number of defined frameshift and nonsense mutations upstream of the galT/galK intercistronic boundary. This has allowed us to manipulate precisely the sites at which upstream translation terminates with respect to the galK initiation codon. The results obtained with these constructed mutations confirm that galT and galK translation are naturally coupled and allow us to assess the consequences of upstream translation on the expression of an adjacent gene.

3. Earlier work utilizing defined bacterial and phage mRNAs transcribed in vitro from a variety of lambdoid phages, investigated the relationship between the modification of 5' triphosphate end of these transcripts with a 7-methyl-guanosine moiety (i.e. "cap" structure) and the translational efficiency of these prokaryotic mRNAs in wheat germ cell-free extracts. Our results demonstrated an almost absolute requirement for the "cap" structure to obtain efficient translation of the prokaryotic transcripts. All the structural information necessary for proper and efficient recognition and translation of prokaryotic mRNAs using eukaryotic components is encoded in the prokaryotic transcript except for the presence of the 5' 7-methyl-guanosine modification. This implies strong evolutionary constraints on the RNA structure which is used for ribosome recognition and translation initiation, as well as similar constraints on those parts of the ribosome which must interact with this RNA structure.



We have now been able to achieve efficient expression of the *E. coli* galactokinase gene directly within mammalian cells. The *Escherichia coli* galactokinase gene (*galK*) was inserted into a modified early region transcription unit of simian virus 40 (SV40) contained on a bacterial plasmid. Introduction of this pSVK vector into monkey, mouse, and hamster cell lines by transfection resulted in efficient expression of the bacterial *galK* gene. This expression was shown to be dependent upon fusion of the *galK* gene to the early promoter of SV40 and did not appear to require SV40 splice signals. Moreover, expression in these cells could be obtained either transiently, 24-72 hr after transfection, or continuously, after stable transformation. In particular, pSVK-dependent *galK* expression was obtained in a hamster cell line genetically deficient in galactokinase activity. Expression of the bacterial enzyme was shown to complement the galactosemic defect of these cells, thereby allowing their selective survival and growth on galactose as the only carbon source. The ability to readily assay, select for, and potentially select against *galK* expression from pSVK and its derivatives should prove extremely useful in studying eukaryotic gene regulatory signals.

4. The lambda *cII* gene product is known to be required *in vivo* for transcriptional activation of both the lambda repressor gene (*cI*) and the integrase function (*int*). This activation is essential for phage lysogenic development.

We have selectively cloned the lambda *cII* gene onto a pBR322 derivative such that *cII* expression is under the control of the lambda  $P_L$  promoter. Transformation of this plasmid into certain bacterial hosts results in high level expression and accumulation of the *cII* product (~1% of total cellular protein). This single polypeptide product was purified to > 95% homogeneity (in mg quantities).

Standard *in vitro* transcription reactions were then carried out in the presence and absence of the purified *cII* protein using as templates appropriate lambda DNA fragments which contain the promoter sites for repressor ( $P_{RE}$ ) and integrase ( $P_I$ ) synthesis. Polyacrylamide gel and fingerprint analyses were used to characterize the RNA transcription products and their 5' start-sites. The results indicate that *cII* protein alone allows RNA polymerase to bind selectively and initiate transcription from these two promoter sites. In the case of the  $P_{RE}$  promoter, *cII* dependent polymerase binding and transcription were eliminated by using DNA templates which contained various *cY* point mutations.

We have purified the phage lambda transcriptional activator protein *cII*. The procedure described allows *cII* to be obtained in both high purity and yield, and thus allows detailed physical and chemical analysis. We demonstrate that *cII* in solution is a tetrameric protein and that it undergoes specific processing at its N-terminal end. In addition the protein is characterized as to its molar extinction coefficient, molecular weight, amino acid composition, isoelectric point,  $\alpha$ -helical content, and antigenic capability.



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Summary Statement  
Laboratory of Molecular Biology  
DCBD, NCI  
October 1, 1981-September 30, 1982

Research in the Laboratory of Molecular Biology focuses on understanding the factors that control gene expression in animal cells and bacterial cells. This information is used to define the biochemical basis for the abnormal growth and behavior of cancer cells. In addition, there is a strong emphasis on investigating the role of the plasma membrane in receiving signals from hormones, growth factors, the extracellular matrix, and other cells and on determining how these signals are transmitted to the genetic apparatus.

Gene Activity and Malignant Transformation

To understand how Rous sarcoma virus transformation alters cell growth and gene expression, N. Richert and D. Blithe have purified to near homogeneity the transforming protein from Rous sarcoma virus induced tumors. Using this purified enzyme S. Ito has shown that the phosphorylation of vinculin, a physiological substrate for the enzyme, is greatly stimulated by the presence of membrane phospholipids, particularly anionic phospholipids such as phosphatidylinositol. This finding indicates that the association of the enzyme with the plasma membrane as shown previously by M. Willingham and I. Pastan is necessary to direct the activity of the enzyme towards specific substrates. An unexpected finding was the observation by N. Richert that the tyrosine-specific protein kinase could also phosphorylate glycerol. This raises the possibility that the enzyme may phosphorylate other intracellular substrates.

The activity of some collagen genes are under the control of Rous sarcoma virus transformation. B. de Crombrughe and coworkers have made considerable progress in isolating various collagen genes. In addition to isolating the  $\alpha 2$  Type I collagen gene they have now isolated the Type III collagen gene. This is also a large gene with approximately 50 exons and 50 introns. Comparison of the structure of the two genes indicates that they probably evolved from a common ancestral gene at least 200 million years ago. C. McKeon has examined the amount of methylation and sensitivity to nuclease digestion of the  $\alpha 2(I)$  collagen gene in active and inactive tissues. She has found that the collagen gene is different from other genes previously studied in its regulation in at least three ways. One is that the promoter region of the collagen gene is not methylated whether or not the gene is expressed. A second is that the gene contains DNAase sensitive sites that are independent of the state of methylation of the gene. The third is that RSV transcription regulates activity of the gene without affecting the DNAase hypersensitive sites. Thus this collagen gene does not follow the rules so far established for the regulation of the activity of other genes. This indicates that malignant transformation may regulate gene activity in a unique manner.

To study regulation of gene activity B. Howard has created new plasmid expression vectors which combine elements of the plasmid PBR322 and the virus SV40 with portions of cellular genes. A particularly useful construction is the insertion of the bacterial chloramphenicol acetylase gene downstream from promoters of genes of interest. Those promoters that have been studied by B. Howard and C. Gorman are the SV40 promoter, the promoter of the avian sarcoma virus and the promoter of collagen. The advantages of the system are that the

enzyme chloramphenicol acetylase is not a normal product of animal cells, and the enzyme is easily assayed in a very sensitive manner. Therefore, even when a small amount of the enzyme is expressed in animal cells, it can be readily detected. In an important recent set of experiments they have shown that the avian sarcoma virus promoter is the most effective promoter yet found for expression of chloramphenicol acetylase in recipient cells after gene transfer. The potency of this promoter probably explains why other related avian tumor viruses can cause B cell lymphomas when inserted at the proper locus in chickens. Using such a vector H. Ohkubo has inserted the collagen promoter adjacent to the chloramphenicol acetylase gene and begun to examine the expression of the collagen promoter when introduced into animal cells. He has found that the 72 base pair repeat of SV40 is a very effective enhancer of collagen expression and currently C. McKeon is searching for enhancing sequences within or close to the collagen gene. At the same time as these in vivo experiments are being pursued, G. Merlino and J. Tyagi have developed a useful cell-free system to investigate collagen gene expression. They have been able to achieve successful transcription of collagen DNA using extracts of Rous sarcoma virus transformed chicken cells. This is the first time that host gene has been transcribed in a homologous in vitro transcription system. Their success was due to a modification of the assay system in which the transcripts synthesized in the cell-free system were analyzed by either primer extension or S1 mapping methods rather than the previously used run-off transcription system. One limiting factor is that normal chick embryo fibroblast extracts are still inactive, whereas the RSV transformed extracts are very active.

Another gene whose activity is markedly lowered by RSV transformation is the fibronectin gene. H. Hirano has now isolated genome clones containing the entire fibronectin gene. Unexpectedly, this gene like the collagen genes is very large being 40 to 50 KB in length and contains many intervening sequences.

M. Gottesman and coworkers have continued their genetic and biochemical studies on the regulation of the growth and shape of Chinese hamster ovary fibroblasts. They have isolated tubulin mutants in CHO cells located in both the genes for  $\alpha$  and  $\beta$  tubulin genes and have found that these mutations affect cell growth by affecting mitotic spindle assembly. C. Roth has found that Rous sarcoma virus transformed CHO cells are resistant to cyclic AMP treatment. Apparently RSV transformation bypasses the pathway by which cyclic AMP usually exerts its actions and slows cell growth. In addition, I. Abraham has developed gene transfer techniques that enables her to transfer genes into CHO cells. The successful development of these methods is of great importance because CHO cells are the most frequently used cell type for genetic studies and previously have been resistant to the transfer of genes. In addition, M. Gottesman has pursued his observation that mouse retrovirus transformed fibroblasts synthesize and secrete a 35,000 molecular weight glycoprotein in very large amounts. This protein (MEP) is primarily located in lysosomes in normal cells but its biological function is unknown.

#### Membrane Molecular Biology

M. Willingham, I. Pastan and collaborators have continued their work on investigating the interaction of growth factors and hormones with the plasma membrane of cells and their subsequent intracellular journey. They have shown that the major pathway by which ligands that bind to specific receptors are internalized is first through clathrin-coated pits and next into a specialized



vesicle that they have identified and named a receptosome to indicate its role in receptor-mediated endocytosis. Entry of molecules through this pathway allows them to enter the cell without being directly routed to lysosomes and rapidly destroyed. Substances that enter the cell by this pathway include epidermal growth factor,  $\alpha_2$ -macroglobulin ( $\alpha_2M$ ), low-density lipoprotein, transferrin,  $\beta$ -galactosidase, vesicular stomatitis virus, Rous sarcoma virus, adenovirus, many other viruses and toxins. Some ligands such as epidermal growth factor and  $\alpha_2$ -macroglobulin are carried by the receptosome from the cell surface into the Golgi region. Using epidermal growth factor coupled to horseradish peroxidase (EGF-HRP) they have followed the growth factor as it traverses the specialized reticular portion of the Golgi, where it becomes concentrated in coated pits of the Golgi. These Golgi-coated pits are half the size of those at the cell surface but appear to function like those at the cell surface to concentrate ligands. From the coated pits of the Golgi, EGF is next transferred to lysosomes. Not all ligands that enter through coated pits and receptosomes complete this route to lysosomes. Viruses such as vesicular stomatitis and adenovirus escape from the receptosome into the cytoplasm. Transferrin enters the Golgi but then is directed back to the cell surface probably through the exocytic pathway.

One important question in studying internalization is whether receptors accompany the ligand into the cell. In collaborative studies with G. Sahagian using an antibody to  $\beta$ -galactosidase, they have shown that  $\beta$ -galactosidase enters the cell with its receptor; both are found in receptosomes. Currently work on the internalization of other receptors using nonspecific antibodies or monoclonal antibodies is in progress.

In related studies the pathway of exocytosis has been investigated using antibodies to the G protein of vesicular stomatitis virus. During the exocytosis of VSV, G protein passes through the Golgi region to the plasma membrane, but not through the coated pits of the Golgi. This directly shows the exocytic pathway does not involve the coated structures of the Golgi. The precise pathway by which G protein leaves the Golgi and appears at the cell surface is unknown.

The steps in ligand internalization involve binding to cell surface receptors, clustering of receptor-ligand complexes in coated pits and formation of a receptosome from a coated pit. The biochemical processes involved in these pathways are not known. R. Dickson and R. Schlegel have found that various ionophores, particularly proton ionophores inhibit endocytosis, and the presence of sodium and bicarbonate in the medium are required for efficient endocytosis. These substances do not interfere either with ligand binding or ligand clustering in coated pits. These treatments may slow or prevent the formation of the receptosome from coated pits. These new findings and earlier studies suggest that the neck of the coated pit transiently closes and some type of an ion pump increases the isosmotic pressure within the "cryptic" coated pit producing a pressure sufficient to generate the receptosome. Previously it was believed that coated pits pinch off to form coated vesicles and "clathrin" from coated vesicles returns to the cell surface to help form new coated pits.

In related studies S. Cheng and R. Horiuchi have investigated the binding of triiodothyronine ( $T_3$ ) to cultured cells and made the unexpected finding that there are a large number of thyroid hormone specific receptors on the surface of cultured cells. Recently they have identified the plasma membrane receptor as a 55,000 molecular weight protein. They have also shown that the receptor-

mediated pathway is the important pathway for thyroid hormone entry. Their findings are of great interest because for more than 20 years it has been believed that thyroid hormones enter cells by directly dissolving in and out of the lipid bilayer.

K. Yamada and coworkers have pursued their studies on fibronectin, a transformation sensitive cell surface glycoprotein. This major cell surface glycoprotein is often decreased on tumor cells and in part accounts for their faulty adhesion. He has investigated the structure and functions of cellular fibronectin and a closely related protein, plasma fibronectin. Although each had the same complement of functional domains for binding to the plasma membrane, collagen, heparin, actin, and DNA, they had at least three sites of apparent polypeptide difference. The plasma membrane binding sites for fibronectin may involve gangliosides or related molecules. Modulation of fibronectin functional activity by physiological concentrations of calcium was also demonstrated. Experiments with various collaborators have suggested a role for fibronectin in mesenchyme cell movement and demonstrated specificity for only one region of fibronectin in mediating chemotaxis. K. Olden has continued studies on the function of carbohydrates moieties in glycoproteins using fibronectin as a typical protein. He has shown that the carbohydrate component plays an important role in the stabilization of specific domains of the protein against proteolytic digestion; however, the carbohydrate does not prevent overall proteolytic attack.

One important enzyme that is involved in cell growth regulation is adenylate cyclase. G. Johnson and co-workers have been studying its regulation by 2-pyridine carboxylic acid, a potent growth inhibitor. They have found that the activity of another membrane associated enzyme, GDP kinase, is greatly increased by treating cells with that compound. This finding indicates that adenylate cyclase may be regulated by other nucleotide metabolizing enzymes in the membrane. This is of particular interest because of the fact that some transforming proteins are nucleotide binding proteins.

S. Wollman and co-workers have developed a culture system to investigate the development and function of the thyroid gland. Using such cultured thyroid cells, they have found that laminin will induce the formation of the basal lamella, an important component of thyroid follicles and many other tissues. This finding will permit them to investigate the mechanism of the formation of the basal lamella, an important organizing element in tissue development.

### Gene Regulation in Prokaryotes

Because cancer cells are characterized by deranged control of synthesis of some proteins and of cell division, frequently a result of viral take-over of specific host machinery, the Laboratory of Molecular Biology also conducts basic research to understand the control mechanisms of protein synthesis, cell division and host-virus interactions using prokaryotic cells as model systems.

S. Adhya and coworkers are studying the modulation of the inducible synthesis of galactose metabolizing enzymes. To characterize the complete regulatory features of the galactose operon, they have now identified the in vivo transcription initiation sites corresponding to the two promoters (PG<sub>1</sub> and PG<sub>2</sub>) that they identified previously, both by sizing the mRNAs and by capping the triphosphate ends with labeled <sup>32</sup>P-GTP and sequencing the labeled RNase T1



fragments. M. Irani has developed a novel in vivo DNA binding assay of the galactose repressor molecules which should be applicable to other systems. Using this technique, M. Irani and L. Orosz have isolated a new set of galactose operator mutants (O's), one of which is located within the first structural gene. This is the first demonstration of a functional operator located inside a structural gene. The biochemical mechanism of this operator element is currently being studied.

S. Gottesman, S. Wickner and J. Auerbach are studying the mechanisms by which the phage  $\lambda$  DNA integrates into or excises from a specific site of the host DNA. The phage proteins, integrase and excisionase, and host factors required for such site specific recombinations have been purified. The integrase and excisionase bind specifically to their corresponding DNA substrates.

M.E. Gottesman, D. Court and collaborators have shown that the cII protein of  $\lambda$  stimulates transcription of the phage integrase and repressor genes from the p<sub>I</sub> and p<sub>RE</sub> promoters, respectively. The stimulation is effective only at low concentrations of cII and does not take place when the cII concentrations are increased suggesting that the cII protein is effective only as a monomer or lower oligomer. G. Guarneros and D. Court have additionally demonstrated a post-transcriptional regulation of the integrase gene at the level of RNA processing. The process is called retroregulation by which transcription of a genetic locus beyond the integrase gene triggers a RNaseIII mediated degradation of the integrase mRNA.

Bacteriophage  $\lambda$  when expressed from a prophage causes several changes in host cell physiology. S. Adhya and M.E. Gottesman have shown that one of these is the expression of neighboring host genes due to extension of transcription from the phage promoters into these genes caused by overcoming the normal transcription termination signals in genes by phage antitermination factor N. By genetic and biochemical techniques, D. Ward, M.E. Gottesman and S. Adhya, in collaboration with D. Friedman, have identified several proteins which participate in cellular transcription reactions, eg., Rho, L, NusB and ribosomal proteins L11 and S10. They are studying how these proteins interact with each other and with phage N protein to effect transcription termination and antitermination and have already shown that NusB and L11 specifically interact with L protein for antitermination purposes.

Several regulatory genes are known in *E. coli* whose products regulate a range of other functions, including cell division, e.g., genes which code for the synthesis of cyclic AMP and its receptor protein (needed for the synthesis of many inducible proteins), Rho factor, Lon protease, and Sula (inhibitor of cell septation). A change in the level of these molecules or mutation in their genes have pleiotropic effects. To understand how these molecules act and how their level are controlled, S. Adhya, S. Gottesman and collaborators have cloned their corresponding genes, are characterizing the products, and are studying their regulation in vivo by fusing their promoters to the easily assayable  $\beta$ -galactosidase structured gene and in vitro by using purified DNA and transcription proteins. S. Garges has shown that the level of cyclic AMP, its receptors protein and Rho are autogenously regulated at the genetic level. S. Mizusawa has found that the level of the Sula protein is modulated by Lon protease at the level of protein stability. Sula concentrations modulate cell division.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08000-12 LMB																								
PERIOD COVERED October 1, 1981 through September 30, 1982																										
TITLE OF PROJECT (80 characters or less)  Regulation of Gene Activity by Cyclic Nucleotides and Transforming Proteins																										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																										
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 60%;">Ira Pastan, Chief, Laboratory of Molecular Biology</td> <td style="width: 10%;">LMB</td> <td style="width: 10%;">NCI</td> </tr> <tr> <td></td> <td>Benoit de Crombrughe, Chief, Gene Regulation Section</td> <td>LMB</td> <td>NCI</td> </tr> <tr> <td>Others:</td> <td>Glenn Merlino, Guest Worker</td> <td>LMB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Jaya Sivaswami Tyagi, Research Fellow</td> <td>LMB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Jose Castano, Visiting Fellow</td> <td>LMB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Hideyasu Hirano, Visiting Fellow</td> <td>LMB</td> <td>NCI</td> </tr> </table>			PI:	Ira Pastan, Chief, Laboratory of Molecular Biology	LMB	NCI		Benoit de Crombrughe, Chief, Gene Regulation Section	LMB	NCI	Others:	Glenn Merlino, Guest Worker	LMB	NCI		Jaya Sivaswami Tyagi, Research Fellow	LMB	NCI		Jose Castano, Visiting Fellow	LMB	NCI		Hideyasu Hirano, Visiting Fellow	LMB	NCI
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COOPERATING UNITS (if any)  None																										
LAB/BRANCH Laboratory of Molecular Biology																										
SECTION Office of the Chief																										
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205																										
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:																								
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																										
SUMMARY OF WORK (200 words or less - underline keywords)  Using a genomic clone containing the promoter of $\alpha 2(I)$ collagen transcription in cell-free extracts has been carried out and the location of the start site of collagen established. Conditions have been established to measure collagen transcription in extracts of chicken cells. Genomic clones containing the entire fibronectin gene have been obtained.																										

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: To understand how cyclic AMP and malignant transformation control gene activity and alter the behavior of cultured cells.

Methods Employed: Prepare cloned cDNAs and isolate genes to use to study messenger RNA synthesis in intact cells or cell-free systems. Isolate animal cells that have mutations in the control of RSV expression and determine the biochemical basis of these alterations.

Major Findings: G. Merlino and J. Tyagi have utilized cell-free extracts of HeLa cells and RSV-transformed chick embryo fibroblasts (RSV-CEF) to study transcription of the 5' part of the gene for  $\alpha 2(I)$  collagen. This has enabled them to determine the location of the promoter of this collagen gene. Collagen expression in RSV-CEF extracts could only be detected using primer extension or S1 protection with a DNA template containing pBR322 sequences downstream from the site of initiation of transcription. To do this, the pBR sequences were labeled with  $^{32}\text{P}$  and therefore endogenous RNA did not interfere with the detection system. Using this very sensitive approach it has not yet been possible to detect transcription in extracts prepared from normal cells, although extracts from other types of transformed cells such as CHO cells can be shown to support transcription.

J. Tyagi has detected and partially purified a low molecular weight factor that prevents the accumulation of RNA polymerase 2 catalyzed transcripts in cell-free extracts. We are now trying to determine the nature of the factor and its mechanism of action.

H. Hirano has used a cDNA derived from the 3' end of the fibronectin gene to screen a chicken genomic library. He has obtained five overlapping clones that contain the entire fibronectin gene. Like collagen the fibronectin gene is very large (>40 kb) and contains many introns and exons.

Significance for Cancer Research and the Program of the Institute: The invasive properties of tumor cells may be related to alterations in collagen, fibronectin and cyclic AMP. Understanding how the synthesis of these molecules is altered in transformation may suggest new methods of cancer treatment.

Publications:

Merlino, G.T., Vogeli, G., Yamamoto, T., de Crombrughe, B., and Pastan, I.: Accurate *in vitro* transcriptional initiation of the chick  $\alpha 2$  (type I) collagen gene. J. Biol. Chem. 256: 11251-11258, 1981.

de Crombrughe, B. and Pastan, I.: Structure and regulation of a collagen gene. TIBS 7: 11-13, 1982.

Vogeli, G., Ohkubo, H., Sobel, M.E., Yamada, Y., Pastan, I., and de Crombrughe, B.: Structure of the promoter for the chick  $\alpha 2$  type I collagen gene. Proc. Natl. Acad. Sci. USA 78: 5334-5338, 1981.

McKeon, C., Ohkubo, H., Pastan, I., and de Crombrughe, B.: Unusual methylation pattern of the alpha 2(I) collagen gene. Cell, in press.

Merlino, G.T., Tyagi, J.S., de Crombrughe, B., and Pastan, I.: Transcription of the chicken  $\alpha 2$  (I) collagen gene by homologous cell-free extracts. J. Biol. Chem., in press.

Vogeli, G., Ohkubo, H., Avvedimento, V.E., Sullivan, M., Yamada, Y., Mudryj, M., Pastan, I., and de Crombrughe, B.: A repetitive structure in the chick  $\alpha 2$  collagen gene. Cold Spring Harbor Symposia on Quantitative Biology 45: 777-783, 1981.

Pastan, I., Willingham, M., de Crombrughe, B., and Gottesman, M.M.: Aging and Cancer: Cyclic AMP and Altered Gene Activity. In Proceedings International Symposium on Aging and Cancer. JNCI-Monograph, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08001-12 LMB																																								
PERIOD COVERED October 1, 1981 through September 30, 1982																																										
TITLE OF PROJECT (80 characters or less)  Role of Cyclic AMP and Transforming Viruses in the Regulation of Cell Behavior																																										
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COOPERATING UNITS (if any) None																																										
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SECTION Office of the Chief																																										
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205																																										
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:																																								
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																																										
SUMMARY OF WORK (200 words or less - underline keywords)  To learn how <u>Rous sarcoma virus</u> transforms cells we have purified the transforming protein from tumors. We are currently characterizing the highly purified protein and assessing its effects when injected into normal cells.																																										



PHS-NIH

Individual Project Number

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: To understand the role of cyclic AMP, hormones and viral gene products in malignant transformation and particularly how these factors regulate the growth, morphology, and other properties of cultured fibroblastic cells.

Methods Employed: Cell culture, viral transformation and standard biochemical analyses of the enzymes involved in cyclic AMP metabolism and the synthesis of macromolecules. Preparations of antibodies against tumors induced by Rous sarcoma virus. Purification of proteins.

Major Findings: The transforming proteins from RSV induced rat tumors has been purified 7,000-10,000 fold by N. Richert and D. Blithe. The purified protein (p54) gives one band on SDS gel electrophoresis with an apparent molecular weight of 54,000 indicating it to be a breakdown product of p60. Using casein as a substrate, N. Richert has characterized some of the kinetic parameters and the nucleotide specificity of the enzyme. One unexpected finding is that the enzyme will phosphorylate glycerol to form glycerol phosphate.

S. Ito has studied the phosphorylation of vinculin by p54. Vinculin is a physiological substrate of the enzyme in cells whereas casein is not. Vinculin is a poor substrate for the purified enzyme but becomes a good substrate in the presence of various anionic phospholipids (phosphatidylinositol, phosphatidylserine and phosphatidic acid). At the same time these phospholipids inhibit the phosphorylation of casein and other non-physiological substrates. We have also investigated the ability of some membrane reactive drugs on the ability of p54 to phosphorylate vinculin. Chlorpromazine and related drugs are effective inhibitors of vinculin phosphorylation.

C. Roth has investigated the response of RSV-transformed CHO cells to cyclic AMP. He finds that these cells are somewhat resistant to the morphologic and growth-inhibiting properties of the nucleotide. This is not due to a change in cyclic AMP dependent protein kinase activity but apparently to a later step in the cyclic AMP pathway.

Significance for Cancer Research and the Program of the Institute: Objective 3, Approaches 1, 2 and 5; Objective 4, Approach 2; and Objective 6, Approach 3.

Various aspects of this work will lead to a better understanding of how cells become cancer cells and how the growth of cancer cells is controlled. It also has therapeutic implications.

Purposed Course: (1) Further characterization of the transforming protein of RSV, and (2) search for cellular targets to account for p60<sup>src</sup> action.

Publications:

Richert, N.D., Blithe, D.L., and Pastan, I.: Properties of the src kinase purified from rous sarcoma virus induced rat tumors. J. Biol. Chem., in press.

Blithe, D.L., Richert, N.D., and Pastan, I.H.: Purification of a tyrosine-specific protein kinase from rous sarcoma virus induced rat tumors. J. Biol. Chem., in press.

Ito, S., Richert, N., and Pastan, I.: Phospholipids stimulate the phosphorylation of vinculin by the tyrosine-specific protein kinase of Rous Sarcoma Virus. Proc. Natl. Acad. Sci. U.S.A., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08006-11 LMB																		
PERIOD COVERED October 1, 1981 through September 30, 1982																				
TITLE OF PROJECT (80 characters or less)  Control of Gene Expression in Bacteriophage Lambda																				
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																				
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 50%;">Max E. Gottesman</td> <td style="width: 40%;">LMB NCI</td> </tr> <tr> <td></td> <td>Head, Biochemical Genetics Section</td> <td></td> </tr> <tr> <td>Others:</td> <td>Douglas Ward, Visiting Fellow</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Amos Oppenheim, Visiting Scientist</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Susan Gottesman, Research Chemist</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Karin Hammer-Jespersen, Guest Worker</td> <td>LMB NCI</td> </tr> </table>			PI:	Max E. Gottesman	LMB NCI		Head, Biochemical Genetics Section		Others:	Douglas Ward, Visiting Fellow	LMB NCI		Amos Oppenheim, Visiting Scientist	LMB NCI		Susan Gottesman, Research Chemist	LMB NCI		Karin Hammer-Jespersen, Guest Worker	LMB NCI
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COOPERATING UNITS (if any)  None																				
LAB/BRANCH Laboratory of Molecular Biology																				
SECTION Biochemical Genetics Section																				
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205																				
TOTAL MANYEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0.0																		
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																				
SUMMARY OF WORK (200 words or less - underline keywords)  We are continuing our study on the nature of transcription termination in <u>E. coli</u> and of the mechanism of action of the bacteriophage <u>lambda antitermination-function</u> , the product of the lambda <u>N-gene</u> . In addition, we are studying the properties of the lambda <u>transcription initiation</u> function, the product of gene <u>cII</u> .																				

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: The lambda gene N-product suppresses transcription termination. Gene cII product stimulates transcription initiation. Our intention is to determine the mechanisms of action of N-function and of cII product.

Methods Employed: Standard microbial genetic and biochemical technique, as well as recombinant DNA technology.

Major Findings: We have been investigating the mechanism of action of the lambda antitermination function, the N-gene product. Our approach involves studying host mutants that affect N activity.

(1) The E. coli NusA protein is required for N-promoted transcription antitermination. Starting with a nusA1<sup>-</sup> mutant, we have isolated host suppressor mutations in which lambda can produce active N-product. One of these resides in the gene encoding the ribosomal large subunit protein, L11. The other mutations are located in the E. coli nusB gene. The nusB mutations are N-specific, i.e., mutations that permit the growth of lambda do not suppress nusA1 for the growth of phage 21, which has a different N-gene than lambda. We have isolated similar nusB mutations that work with the N-product of 21, but not with lambda. The mutations appear to be located in different portions of the nusB gene, suggesting that the NusB protein may have two domains, one recognizing lambda N-protein (or the polynucleotide sequence with which lambda N-protein interacts) and the other recognizing 21 N-protein.

(2) A particular class of rho mutants, defective for T4 growth, are also defective for growth of lambda. We have isolated suppressors of these mutations which restore the activity of lambda N protein. Some of these suppressors are located near the lambda prophage probably in gene N, and display N a specificity for mutant Rho factor. This suggests the existence of an interaction between N-protein and Rho, and gives some hints as the mechanism of transcription antitermination by N-protein.

We find that the cII protein of lambda stimulates the lambda pI and pE promoters at low concentrations but inhibits their activity at higher levels. Induction of the E. coli lac operon is inhibited strongly by cII-protein at all concentrations tested. cII-protein appears to kill E. coli, and we have isolated RNA polymerase mutants that prevent this killing. In addition, we have obtained RNA polymerase mutants that partially obviate the cII requirement for pE activity.

Significance for Cancer Research and the Program of the Institute:

National Cancer Plan Objective 3, Approach 1. In cancer cells, the expression

of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using  $\lambda$  as a model system. This understanding might help to prevent the conversion of normal cells to those capable of forming cancers.

Proposed Course: (1) To study other host mutations that influence the activity of N-gene product. (2) To isolate and sequence additional N-gene mutants that interact specifically with mutant host proteins. (3) To understand the regulation of cII-protein synthesis and activity. (4) To understand the biochemistry of transcription initiation and termination signals.

Publications:

Ward, D.F., and Gottesman, M.E.: The nus mutations affect transcription termination in E. coli. Nature 292: 212-215, 1981.

Greenblatt, J., Li, J., Adhya, S., Friedman, D., Baron, L., Redfield, B., Kung, H., and Weissbach, H.: Evidence that the L factor required for DNA dependent In vitro synthesis of  $\beta$ -galactosidase is the E. coli nusA gene protein. Proc. Natl. Acad. Sci. USA 77: 1991-1994, 1980.

Gottesman, M., Adhya, S., and Das, A.: Transcription antitermination by lambda N-gene product. J. Mol. Biol. 140: 57-75, 1980.

Ward, D.F., and Gottesman, M.E.: Suppression of transcription termination by phage lambda. Science, 1982, in press.

Oppenheim, A., Gottesman, S., and Gottesman, M.E.: Regulation of  $\lambda$  int Gene Expression. J. Mol. Biol., 1982, in press.

Adhya, S., and Gottesman, M.E.: Promoter Occlusion: Transcription through a promoter inhibits its activity. Cell, 1982, in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  201 CB 08010-08 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less) The Morphologic Mechanisms of Motility and Neoplastic Transformation in Culture		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Mark C. Willingham Chief, Ultrastructural Cytochemistry Section		
	LMB	NCI
Others: Ira H. Pastan Chief, LMB		
	LMB	NCI
Jurgen Wehland Staff Fellow		
	LMB	NCI
David FitzGerald Guest Worker		
	LMB	NCI
G. Gary Sahagian Guest Worker		
	GBB	NIAMDD
COOPERATING UNITS (if any)  Genetics and Biochemistry Branch, IRP, NIAMDD		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Ultrastructural Cytochemistry Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4.0	PROFESSIONAL: 4.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Neoplastic transformation produces many changes in cell physiology. We have utilized morphologic techniques to study these changes and to understand certain basic cellular mechanisms of motility, organelle movement, and compartmentalization of intracellular materials. We have employed <u>ultrastructural immunocytochemistry</u> , single cell <u>microinjection</u> , and <u>image intensification</u> techniques to follow the movement of substances through various endocytic and exocytic compartments within the cell, as well as the cytoplasmic structures mediating movements of the entire cell and its subcellular organelles. These include localization of the endocytosis of external macromolecules and the receptors which bind them, particularly those for <u>epidermal growth factor</u> , <u>transferrin</u> , <u>lysosomal enzymes</u> and <u>alpha2-macroglobulin</u> . We have also followed the exocytosis of <u>Vesicular Stomatitis Virus G protein</u> , as well as the transit of epidermal growth factor through the Golgi system. These results have demonstrated that the <u>clathrin-coated pits</u> of the Golgi system are involved in transfer of materials into lysosomes, but are not involved in exocytosis. Preliminary studies have been conducted on the movement of organelles in living cells using microinjection of <u>antibodies with image intensification and electron microscopy</u> .		

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

#### PROJECT DESCRIPTION:

Objectives: To investigate the mechanisms that control cell motility, endocytosis, exocytosis, intracellular protein traffic, viral infection, and the morphologic and growth manifestations of malignant transformation.

Methods Employed: Cell culture, specialized light, and electron microscopic morphologic and immunocytochemical methods, including the EGS ultrastructural localization procedure, single cell microinjection techniques, and specialized biochemical purification and analytical methods.

Major Findings: The movement of cells and subcellular organelles has been directly studied and followed using electron microscopic immunocytochemistry, single cell microinjection, and image intensification techniques. We have previously established the location and organization of a number of structural proteins within cells in an effort to understand their relationships and effects on morphologic alterations followed transformation. These studies included locating the position of specific virally-coded src proteins on the inner aspect of the plasma membrane (ASV p60<sup>src</sup> and Ha-MSV p21<sup>src</sup>) in transformed cells. In addition, we have studied the movements and activities of morphologic structures at the plasma membrane, particularly the clathrin-coated pits of the cell surface which mediate receptor-dependent endocytosis of external growth factors and macromolecules.

We have extended these studies to the processes of endocytosis and exocytosis in cultured cells. Most recently, we have followed the pathway taken by growth factors from the cell surface coated pits into receptosomes, the intracellular endocytic organelles derived from coated pits at the cell surface. We have further followed the transit of epidermal growth factor and  $\beta$ -galactosidase through the Golgi system after delivery from receptosomes into the Golgi, and have found that these ligands selectively cluster in the clathrin-coated pits located in the Golgi prior to delivery to lysosomes. Further, we have followed the transit of Vesicular Stomatitis Virus G protein through the Golgi system. This protein is selectively delivered to the cell surface and is not delivered to lysosomes. We found that G protein did not enter clathrin-coated pits of the Golgi prior to exocytosis. This suggests that these Golgi clathrin-coated pits are involved in sequestration of materials destined for lysosomes and not for exocytosis. Our previous studies had suggested that the clathrin-coated pits of both the plasma membrane and the Golgi are stable structural elements which do not pinch off to form isolated coated vesicles in living cells.

Recently, we have also begun to study the location and movement of intracellular molecules accessible to the cell cytosol by microinjecting antibodies to those proteins. These include injection of anti-tubulin and antibodies directed against the COOH-terminal peptide fragment of VSV G protein accessible on the cytoplasmic face of intracellular membranes. These studies

have been analyzed by fluorescence microscopy of fluorescently-labeled antibody injected into living cells visualized by image intensification techniques, as well as through the use of colloidal gold-labeled antibody injected and then viewed after fixation by electron microscopy. These studies have shown dramatic specific interaction of injected antibodies with their protein-antigens. For example, injection of anti-tubulin results in antibody coating of microtubule and cross-linked bundles of microtubules in the living cell with inhibition of the saltatory motion of intracellular organelles along these microtubules tracks. Injection of antibodies to the COOH-terminal fragment of VSV G protein prior to VSV injection results in interference with the transit of G protein from the endoplasmic reticulum to the Golgi and to the plasma membrane. Such direct experiments are being continued in the hope of understanding the exact nature of the vesicular transport system of exocytosis in these cultured cells.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 6, Approach 3.

Transformation of cultured cells appears closely linked with the ability to form malignant tumors *in vivo*. The understanding of the basic mechanisms that control cell movement, response to growth-promoting factors, virus infectivity, and the morphologic changes that occur following transformation by transforming viruses are likely to be of great value in understanding the basic mechanisms that are altered in most cancer cells. This understanding is likely to have significant impact on the ability to design successful therapeutic procedures.

Proposed Course: We will continue to study the basic cellular morphologic mechanisms that regulate cell movement, entry and exit of cell proteins, viruses, hormones, and the interactions of specific transformation-linked molecules (src proteins) with these systems.

Through the study of the alterations in cell functions related to transformation, we will try to gain a more specific understanding of the precise mechanisms by which cancer cells are able to grow uncontrollably and metastasize.

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Willingham, M.C., Yamada, S.S., Bechtel, P.J., Rutherford, A.V., and Pastan, I.: Ultrastructural immunocytochemical localization of myosin in cultured fibroblastic cells. J. Histochem. Cytochem. 29: 1289-1301, 1981.

Wehland, J., Willingham, M.C., Gallo, M.G., and Pastan, I.: The morphologic pathway of exocytosis of the G protein of vesicular stomatitis virus in cultured fibroblasts. Cell 28: 831-841, 1982.

Willingham, M.C., Pastan, I., Sahagian, G.G., Jourdian, G.W., and Neufeld, E.F.: A morphologic demonstration of the pathway of internalization of a lysosomal enzyme through the mannose 6-phosphate receptor in cultured CHO cells. Proc. Natl. Acad. Sci., U.S.A. 78: 6967-6971, 1981.

Wehland, J., Willingham, M.C., Rutherford, A.V., Gallo, M.G., Rudick, J., and Pastan, I.: The role of clathrin coated structures in the endocytosis and exocytosis of macromolecules in cultured fibroblasts. In Albrecht-Buhler, G., and Watson, J.D. (Eds.): Cold Spring Harbor Symposia. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory, vol. 46, 1982, in press.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08011-08 LMB						
PERIOD COVERED October 1, 1981 through September 30, 1982								
TITLE OF PROJECT (80 characters or less)  Structure and Role of a Transformation-Sensitive Cell Surface Glycoprotein								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 50%;">           PI: Kenneth M. Yamada                  Head, Membrane Biochemistry Section         </td> <td style="width: 50%; text-align: right;">           LMB NCI         </td> </tr> <tr><td colspan="2"> </td></tr> <tr> <td>           Others: Masao Hayashi, Visiting Fellow                      Hideyasu Hirano, Visiting Fellow                      Takayuki Hasegawa, Visiting Fellow                      Steven K. Akiyama, Guest Worker                      Etsuko Hasegawa, Guest Worker         </td> <td style="text-align: right; vertical-align: top;">           LMB NCI            LMB NCI            LMB NCI            LMB NCI            LMB NCI         </td> </tr> </table>			PI: Kenneth M. Yamada Head, Membrane Biochemistry Section	LMB NCI			Others: Masao Hayashi, Visiting Fellow Hideyasu Hirano, Visiting Fellow Takayuki Hasegawa, Visiting Fellow Steven K. Akiyama, Guest Worker Etsuko Hasegawa, Guest Worker	LMB NCI LMB NCI LMB NCI LMB NCI LMB NCI
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COOPERATING UNITS (if any)  Diabetes Branch, NIAMDD								
LAB/BRANCH Laboratory of Molecular Biology								
SECTION Membrane Biochemistry Section								
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205								
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SUMMARY OF WORK (200 words or less - underline keywords)  <p>           The major cell surface glycoprotein fibronectin is often decreased on <u>tumor cells</u> and is involved in <u>cellular adhesion</u>. We have investigated its structure and functions. <u>Cellular fibronectin</u> and the closely related protein plasma fibronectin were compared. Although each had the same complement of functional domains for binding to the <u>plasma membrane</u>, <u>collagen</u>, <u>heparin</u>, <u>actin</u>, and <u>DNA</u>, they had at least three sites of apparent polypeptide difference. According to competitive inhibition studies, the plasma membrane binding sites for fibronectin may involve gangliosides or related molecules. A modulation of fibronectin functional activity by physiological concentrations of <u>calcium</u> was also demonstrated. Collaborative experiments suggested a role for fibronectin in mesenchyme <u>cell movement</u>, demonstrated specificity for only one region of fibronectin in mediating <u>chemotaxis</u>, and characterized the <u>insulin receptor</u> and the process of <u>down-regulation</u>.         </p> <p>           Our objectives will be to determine the structure and organization of the multiple active sites of fibronectin, the mechanisms by which it regulates cell behavior and <u>embryonic differentiation</u>, and the structure and function of its <u>gene</u>.         </p>								



PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: The major cell surface protein fibronectin is depleted after neoplastic transformation. Our objectives are to determine fibronectin's biochemical structure, its role in cell behavior, its mechanism of action, and its regulation.

Methods Employed: Fibronectin is isolated by urea-extraction and gelatin affinity chromatography. Structural and functional domains of cellular and plasma forms of fibronectin are compared by analysis of polypeptide domains resistant to thermolysin and to other proteases using affinity chromatography on a standard series of columns containing ligands such as collagen, heparin, actin, or DNA, by electrophoresis on step-gradient SDS gels, and by one-dimensional peptide mapping.

Competitive inhibitors of the adhesive activities of fibronectin are examined in standardized assays for hemagglutination, attachment of cells to type I collagen, spreading of cells on tissue culture substrates in serum-free medium, and restoration of normal morphology to transformed fibroblasts. For inhibitor studies ganglioside oligosaccharides are produced by ozonolysis, and glycopeptides are produced by pronase digestion and purified by molecular sieve chromatography.

Protein turnover is measured by determining the rate of loss of radioactivity from pulse-labeled proteins immunoprecipitated from cell homogenates and analyzed by SDS polyacrylamide gel electrophoresis.

Major Findings: The glycoprotein fibronectin functions in interactions of cells with a variety of cellular and extracellular macromolecules. There are at least two major forms of this glycoprotein, termed cellular fibronectin and plasma fibronectin. We compared the structure and function of these molecules in detail. There are striking similarities in the organization of protease-resistant functional domains in these glycoproteins, with no detectable differences in domains that bind to the plasma membrane, collagen, heparin, actin, or DNA. However, there are three separate regions of apparent polypeptide difference, suggesting that although these molecules are very similar, they are probably derived from different messenger RNAs and possibly different genes.

The mechanisms by which fibronectin binds to cells in mediating adhesive events was investigated. Competitive inhibition experiments suggest that gangliosides, or molecules with related charge distributions, may be necessary for the interaction of fibronectin with cells in four different adhesive events, including fibronectin-mediated restoration of normal morphology to transformed cells.

The possibility that fibronectin function can be regulated by local environmental cues was explored with human plasma fibronectin. Divalent cations are found to modulate the binding of heparin to fibronectin at two of three sites. Modulation of the binding of heparin to the amino-terminal domain of fibronectin occurs at physiological concentrations of calcium.

We continued collaborative studies of the roles of fibronectin in embryonic development. A classical model system for embryonic cell and tissue movement is the ingression of primary mesenchyme cells preceding sea urchin gastrulation. Fibronectin is found to appear suddenly on the cell surface of these cells at the time of initiation of cell movement. Since tissue culture model studies show that fibronectin stimulates cell migration, these in vivo results suggest a function for membrane-associated fibronectin in developmentally regulated cell movement. Fibronectin also promotes the chemotaxis of fibroblasts, and this activity is found to be mediated by the specific cell-binding region of fibronectin.

The human cell surface receptor for insulin has been characterized in collaborative studies. It consists of several disulfide-linked multimeric complexes with subunits of 95, 135, and 210,000 daltons. The important process of "down-regulation," now known to occur for many cell surface receptors after binding of a ligand, was found to result from an increased rate of protein degradation of the insulin receptor.

Significance for Cancer Research and Program of the Institute: National Cancer Plan Objective 3, Approach 5.

The glycoprotein fibronectin functions in normal cellular adhesion and cell-cell interactions, and its loss from cells of certain tumors may account for some of the abnormal behavior of these cells. Our studies help elucidate the mechanisms by which fibronectin binds to ligands and to cells, the nature of the different forms of fibronectin, its regulation, and its role in normal embryonic development. Studies of the regulation of quantities of fibronectin and other cell surface molecules should provide insights into the pathogenesis of diseases in which they are altered; knowledge of how they act should promote an understanding of the regulation of normal and malignant cell behavior.

Proposed Course: To determine the organization and structure of the as yet poorly understood carboxy terminal half of fibronectin, to further define the membrane component to which fibronectin binds during cell adhesion, to attempt to test the function of fibronectin in vivo by immunological inhibition, and to characterize the gene(s) for fibronectin.

Publications:

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Kasuga, M., Hedo, J.A., Yamada, K.M., and Kahn, C.R. The structure of insulin receptor and its subunits: Evidence for multiple non-reduced forms and a 210K component. J. Biol. Chem., 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08700-10 LMB																																												
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<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">Benoit de Crombrughe</td> <td style="width: 40%;">Chief, Gene Regulation Section</td> <td style="width: 10%;">LMB NCI</td> </tr> <tr> <td colspan="4"> </td> </tr> <tr> <td>Others:</td> <td>Hiroaki Ohkubo</td> <td>Visiting Fellow</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Yoshihiko Yamada</td> <td>Visiting Associate</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Maria Mudryj</td> <td>Chemist</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Rodolfo Frunzio</td> <td>Visiting Fellow</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Catherine McKeon</td> <td>Postdoctoral Fellow</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Azriel Schmidt</td> <td>Visiting Fellow</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Gene Liau</td> <td>Postdoctoral Fellow</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Klaus Kuhn</td> <td>Fogarty Scholar</td> <td>LMB NCI</td> </tr> <tr> <td></td> <td>Ricardo Brentani</td> <td>Visiting Professor</td> <td>LMB NCI</td> </tr> </table>			PI:	Benoit de Crombrughe	Chief, Gene Regulation Section	LMB NCI					Others:	Hiroaki Ohkubo	Visiting Fellow	LMB NCI		Yoshihiko Yamada	Visiting Associate	LMB NCI		Maria Mudryj	Chemist	LMB NCI		Rodolfo Frunzio	Visiting Fellow	LMB NCI		Catherine McKeon	Postdoctoral Fellow	LMB NCI		Azriel Schmidt	Visiting Fellow	LMB NCI		Gene Liau	Postdoctoral Fellow	LMB NCI		Klaus Kuhn	Fogarty Scholar	LMB NCI		Ricardo Brentani	Visiting Professor	LMB NCI
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COOPERATING UNITS (if any) Margery Sullivan, Genex Corp.; Michael O'Neill, University of Maryland, Catonsville, Md.; Bruce Howard & Cory Gorman, NCI; Mark Sobel, NIDR; Bill Uphold, University of Chicago; Moshi Yaniv, Pasteur Institute, Paris France.																																														
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SUMMARY OF WORK (200 words or less - underline keywords) 1. We have isolated two complete chick collagen genes: $\alpha 2$ type I and type III and have begun to isolate three others $\alpha 1$ type I, type II, and type IV. Comparison of the structure of the $\alpha 2$ type I and type III collagen gene has revealed additional information on the assembly mechanism of the ancestral gene, its amplification and the further evolution of the individual genes. Over the 200,000,000 years since their divergence, the genes for $\alpha 2(1)$ and type III have undergone little or no recombinational duplications or rearrangement of their exons except for conversions of small segments of genes. 2. Studies on methylation and DNase sensitivity of the chick $\alpha 2(1)$ collagen gene from several types of tissues with different levels of collagen synthesis suggests at least three levels of regulations for the expression of these genes. 3. We have studied the expression of the promoter region of the chick $\alpha 2(1)$ collagen gene after transfection into chick embryo fibroblast in culture with a tester gene linked to the promoter. The activity of this promoter is increased by "enhancing sequences" like those which contain the 72 bp repeat of SV40 or an analogous region in polyoma. The level of gene expression can also be increased by changing the sequences surrounding the polyA addition site.																																														



PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objective: To understand the differentiation programs which determine the level of expression of specific genes in animal cells.

Methods Employed: (1) Introduction of segments of animal and bacterial genes in the DNA of bacterial plasmids and bacteriophage; construction of recombinant DNAs; construction and screening of cDNA and gene libraries; (2) DNA transfection of animal cells, selection of transfected cells; (3) purification of specific DNA fragments and RNA species; nucleotide sequence analysis of DNA and RNA; (4) measurement of RNA and protein synthesis in vivo and in cell-free systems; (5) purification of factors active in regulation of gene expression.

Major Findings:

1. Structure of the  $\alpha 2$  type I and type III collagen gene. We isolated another chick collagen gene, the type III collagen gene, and have compared its structure with the gene for  $\alpha 2(I)$  collagen which we had previously isolated. The size of the mature  $\alpha 2(I)$  and type III collagen genes are the same and both genes have the same number of exons (approximately 50). Comparison of both the structure and sequences of exons in these two genes confirms our earlier hypothesis that the first ancestral collagen gene arose by amplification of a DNA segment containing a 54 bp exon. These amplifications probably occurred by unequal crossing-over. During or after these amplifications, a number of exon rearrangements occurred leading to the precise excision of some introns and also to the formation of exons with sizes which diverged from 54 or 108 bp by 9 bp or by multiples of 9. This first ancestral gene was probably duplicated several times and gave way to the ancestral gene for the fibrillar collagen in animals. This latter collagen ancestral gene which contains exons for a signal peptide, N-terminal peptide and a C-terminal peptide, was further duplicated to give the genes for  $\alpha 1$  type I,  $\alpha 2$  type I, and type III. These events occurred at least 200 million years ago. At this time, the ancestral collagen gene had acquired optimal number of exons and recombinational amplification of DNA segment containing 54 bp exons were no longer tolerated. Furthermore, few exon rearrangements of the type described above occurred after this time. We have determined the sequence of the four exons coding for the C-terminal peptide of type III collagen. As in the  $\alpha 2(I)$  collagen gene, the exon coding for the N-terminal part of the C-peptide fused to the exon coding for the last part of the helical region. The sizes of the first three exons are similar in both genes. The first exon of type III is larger than the equivalent exon of type I  $\alpha 2$  gene because it contains a larger untranslated segment. The overall homology between the  $\alpha 2$  I and III gene is about 60 per cent for these four exons. There is, however, a segment of 48 bps located in the exon second from the 3' end in which 47 of the 48 bp are conserved. This remarkable homology suggests a recent rearrangement probably due to a gene conversion. Because the same segment is also conserved in the  $\alpha 1(I)$  gene and the type II gene, we suggest that gene conversion could be a very frequent recombinational mechanism in animal cells.

2. Three levels of regulation for the  $\alpha 2(I)$  collagen gene. We have examined the methylation status of three segments of the chick  $\alpha 2(I)$  collagen gene in five different types of tissues with either no collagen synthesis or different levels of synthesis. We find that a segment around the initiation site for transcription is under methylated or unmethylated in tissues examined whether or not they synthesize collagen. In contrast, the body for the gene is methylated in every tissue which we examined. Our results suggest, therefore, that the level of expression of the  $\alpha 2(I)$  collagen gene is independent of methylation. We believe, however, that this absence of methylation around the start site for transcription could have a regulatory function. One hypothesis is that this gene needs to be activated more rapidly during development than a demethylation process would permit.

The sensitivity of chromatin containing  $\alpha 2(I)$  collagen gene to DNase I, was also examined in different tissues. Our results show that the relative sensitivity of methylated and unmethylated segment of this gene is the same within a given tissue. In fibroblasts which express the gene, however, this overall sensitivity is greater than in brain, a tissue that does not express the gene. Fibroblast and fibroblast transformed by the Rous sarcoma virus, but not brain, also contain a site which is hypersensitive to DNase I, located in the promoter region. We conclude that the changes in the DNase I sensitivity are correlated with expression and are independent of the methylation pattern of the gene.

Overall, our results suggest a model with at least three levels of regulation for this gene. A first mechanism keeps a definite segment around the promoter from being methylated and functions in all cell types. A second corresponds to a change in chromatin structure demonstrated by an increased susceptibility to DNase I and presence of a discrete DNase I hypersensitive site near the start of transcription. This mechanism is operative in both normal and RSV-transformed fibroblast in culture and probably in all other cells committed to the expression of the  $\alpha 2(I)$  collagen gene regardless of the actual level of expression. The third mechanism, which is revealed by the inhibitory effect of p60<sup>src</sup> on type I collagen RNA transcription, controls the relative level of expression of the  $\alpha 2(I)$  collagen gene in already committed cells.

3. Expression of the  $\alpha 2(I)$  collagen gene promoter. We have studied the expression of the  $\alpha 2(I)$  collagen gene promoter by DNA transfection of chick embryo fibroblast using recombinant DNA plasmids. To examine its activity, we have linked the collagen promoter to the bacterial gene for chloramphenicol acetyl transferase (CAT). Because we assay the levels of the enzyme 30 to 48 hours after transfection, these experiments examine the "transient" expression of the promoter.

Two types of sequences increase the level of CAT expression. First, sequences present in the regulatory region of SV40 DNA containing the 72 bp repeat enhance the activity of the collagen promoter about 15- to 34-fold. These sequences were cloned in a circular plasmid at a distance of about 2Kb downstream from the collagen promoter. These sequences keep their enhancing role when cloned in either orientation. The sequence present between the early and late promoter of polyoma DNA has a similar effect on the activity of the collagen promoter.

The sequence was cloned in the same site of our vector as the SV40 sequences. We propose that these sequences are entry sites for RNA polymerase molecules, which would be directed from there, eventually, via other specific sequences, to the start site of the transcription. Deletions introduced by in vitro recombination methods around the collagen promoter affect its expression.

Other sequences which can influence the level of expression of a gene in our recombinant plasmid for collagen promoter expression are those surrounding the site of poly A addition. We have replaced the poly A addition site which came from the early region of SV40 by a sequence from the type III collagen gene containing three adjacent functional poly A addition sites. This replacement resulted in a 50- to 100-fold increase in the levels CAT enzyme after DNA transfection. We believe that this sequence could increase the proportion of RNA molecules originating at the collagen promoter, which are transported from the nucleus and translated into protein in the cytosol.

We have also begun to examine the collagen promoter after it has been introduced into mouse T3-cells by co-transfection with a selectable marker. Cells which have acquired a selectable marker by DNA transfection are examined for expression of the collagen promoter. We will analyse the expression of the collagen promoter in mouse cells transformed by RSV and eventually determine which DNA sequences are mediated by P60<sup>src</sup> in collagen synthesis.

#### Significance for Cancer Research and the Program of the Institute:

The collagens are major differentiation products of specific cell types. p60<sup>src</sup> and other viral oncogenic proteins cause a severe reduction of type I collagen synthesis. There is good evidence that this effect, although indirect, is mediated by a transcriptional control mechanism. Our studies will help us understand how the collagen genes are regulated in various cell types and how oncogenic protein can alter this regulation. We should also gain more understanding of the genetic prospects of specific differentiation programs in animal cells.

#### Proposed Course:

1. Study the activity of the promoter of various collagen genes by DNA transfection of cells in culture in both their transient and permanent expression. Examine the sequence specificity of these promoters by introduction of deletions, substitutions and point mutations. Study how the presence in the cells of oncogenic proteins like p60<sup>src</sup> or p21<sup>ki</sup> affect the activity of these promoters.
2. Isolate the promoter regions of several mouse collagen genes. Compare structures and sequences with those of other collagen genes.
3. Develop in vitro assays which reproduce the cell specific activity of a collagen gene promoter, i.e. in vitro transcripton, in vitro reconstitution of DNase I sensitivity or hypersensitivity of chromatin, DNA binding of specific protein.
4. Examine where the activation of a given collagen promoter in a specific

cell type is itself determined by the product of another gene which would direct pleiotropic program of differentiation; attempt the isolation of such gene.

5. Isolate one of the human collagen genes and examine the nature of the defect in some genetically transmitted diseases affecting one of the collagen genes.

6. Obtain additional promoter mutants in the galactose operon and in defined segments of the cyclic AMP receptor gene of the E. coli by site-specific mutagenesis to better understand promoter action of CRP.

#### Publications:

Avvedimento, E., Yamada, Y., Lovelace, E., Vogeli, G., de Crombrughe, B., Pastan, I.: Decrease in the levels of nuclear RNA precursors for  $\alpha 2$  collagen in Rous sarcoma virus transformed fibroblasts. Nucleic Acid Research 9: 1123-1131, 1981.

Sobel, M.E., Yamamoto, T., de Crombrughe, B., and Pastan, I.: Regulation of procollagen messenger ribonucleic acid levels in Rous sarcoma virus transformed chick embryo fibroblasts. Biochemistry 20: 2678-2684, 1981.

O'Neill, M.C., Amass, K., and de Crombrughe, B.: Molecular model of the DNA interaction site for the cyclic AMP receptor protein. Proc. Natl. Acad. Sci. USA 78: 2213-2217, 1981.

Vogeli, G., Ohkubo, H., Avvedimento, V.E., Sullivan, M., Yamada, Y., Mudryj, M., Pastan, I., and de Crombrughe, B.: A repetitive structure in the chick  $\alpha 2$  collagen gene. Cold Spring Harbor Symposia on Quantitative Biology 45: 777-783, 1981.

Merlino, G.T., Vogeli, G., Yamamoto, T., de Crombrughe, B., and Pastan, I.: Accurate *in vitro* transcriptional initiation of the chick  $\alpha 2$  (Type I) collagen gene. J. Biol. Chem. 256: 11251-11258, 1981.

Vogeli, G., Ohkubo, H., Sobel, M.E., Yamada, Y., Pastan, I., and de Crombrughe, B.: Structure of the promoter for the chick  $\alpha 2$  collagen gene. Proc. Natl. Acad. Sci. USA 78: 5334-5338, 1981.

Ohkubo, H., Avvedimento, E., Yamada, Y., Vogeli, G., Sobel, M., Merlino, G., Mudryj, M., Pastan, I., and de Crombrughe, B.: The collagen gene. ICN/UCLA Developmental Genetics. 1981.

Aiba, H., Adhya, S., and de Crombrughe, B.: Evidence for two functional gal promoters in intact Escherichia coli cells. J. Biol. Chem. 256: 11905-11910, 1981.

Busby, S., Irani, M., and de Crombrughe, B.: Isolation of mutant promoters in the Escherichia coli galactose operon using local mutagenesis on cloned DNA fragments. J. Mol. Biol. 154: 197-209, 1982.



Busby, S., Aiba, H., and de Crombrughe, B.: Mutations in the Escherichia coli galactose operon that define two promoters and the binding site of the cyclic AMP receptor protein. J. Mol. Biol. 154: 211-227, 1982.

de Crombrughe, B., and Pastan, I.: Structure and regulation of a collagen gene. TIBS 7: 11-13, 1982.

McKeon, C., Ohkubo, H., Pastan, I., and de Crombrughe, B.: Unusual methylation pattern of the  $\alpha 2(I)$  collagen gene. Cell 29: 203-210, 1982.

Pastan, I., Willingham, M., de Crombrughe, B., and Gottesman, M.M.: Aging and Cancer: Cyclic AMP and Altered Gene Activity. In Proceedings International Symposium on aging and Cancer. JNCI-Monograph, in press.

Merlino, G.T., Tyagi, J.S., de Crombrughe, B., and Pastan, I.: Transcription of the chicken  $\alpha 2(I)$  collagen gene by homologous cell-free extracts. J. Biol. Chem., in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08702-21 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less) Endocytosis in the Thyroid Gland		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI:            Seymour H. Wollman      Chief, Cell Organization Section      LMB    NCI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Cell Organization Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.0	PROFESSIONAL: 0.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  The typical thyroid epithelial cell can take in <u>colloid</u> from the <u>follicular lumen</u> by <u>macropinocytosis</u> . It can also phagocytose red blood cells. We propose to study the <u>mechanism</u> of these processes by electron microscopy, histochemistry, and related techniques.		

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: To determine mechanism of thyroid hormone release and, more generally, endocytotic properties of typical thyroid epithelium.

Methods Employed: Project was not pursued this year.

Significance for Cancer Research and the Program of the Institute: This project fits most clearly under Objective 3, Approach 5, having to do with properties of the cell surface.

Proposed Course: To be continued.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08703-30 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less) Thyroid Hormone Synthesis and Storage		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI:            Seymour H. Wollman      Chief, Cell Organization Section      LMB    NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Cell Organization Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.0	PROFESSIONAL: 0.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <p style="margin-left: 40px;">             This project has as its objectives to learn about: (1) The mechanism by which the <u>thyroid gland</u> maintains a concentration of iodide elevated above that of the blood, and (2) The mechanism by which the thyroid gland forms and accumulates protein-bound iodine.           </p> <p style="margin-left: 40px;">             The work is largely based upon <u>in vivo</u> studies and makes use of autoradiography, cytochemistry at ultrastructural level, kinetic studies, and inhibitors of steps in the synthesis of thyroid hormones.           </p>		

PHS-NIH  
Individual Project Report  
October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: To learn about the mechanism of synthesis and storage of thyroid hormones.

Methods Employed: Not worked on this year.

Significance for Cancer Research and the Program of the Institute:  
Some of the work reported here may best be put into Objective 3, Approach 5 because they give information on properties of cell surface and cell membranes.

Proposed Course: To be continued.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08704-30 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less) Thyroid Growth and Involution		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Seymour H. Wollman Chief, Cell Organization Section LMB NCI  Others: Osamu Tachiwaki Visiting Fellow LMB NCI Corrado Garbi Visiting Fellow LMB NCI Staffan Smeds Visiting Scientist LMB NCI		
COOPERATING UNITS (if any) Lucio Nitsch, Istituto di Patologia Generale, Universita di Napoli, Naples, Italy Jean Pierre Herveg, Faculte de Medecine, Catholic University of Louvain, Brussels, Belgium		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Cell Organization Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4.0	PROFESSIONAL: 3.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  To study the thyroid growth and the production and properties of trans-plantable <u>thyroid tumors</u> in the rat: changes occurring during growth, <u>hyperplasia</u> and <u>involution</u> , growth and properties of thyroid cells and thyroid tumor cells in culture.		



PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: To study thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring in the thyroid during growth, hyperplasia and involution, factors controlling thyroid differentiation.

Methods Employed:

Hyperplasia: Hyperplasia was produced in rat thyroid gland by feeding the goitrogen, thiouracil. Various details of the nature of the growth process were examined by light and electron microscopy.

Involution: Involution of the hyperplastic thyroid gland was induced by removing the diet containing a goitrogen and feeding the rats a diet with high iodine content.

Tissue Culture: Thyroid gland was dissociated by collagenase treatment. Small clusters of epithelial cells were isolated from the dissociated tissue by centrifugation followed by filtration through fine nylon mesh. Clusters were cultured in suspension and their properties were examined by electron microscopy.

Major Findings:

Tissue culture (with C. Garbi):

1. Induction of a basal lamina on separated thyroid follicles in suspension culture: Thyroid follicles separated from each other by collagenase do not develop a basal lamina in our cultures. Very short segments of a basal lamina form when follicles are embedded in a dilute collagen gel in 0.5% serum. The addition of laminin without collagen results in the deposition of short segments within 30 minutes, and these lengthen as time in culture increases.

2. Stabilization of follicles in suspension culture: Follicles in suspension culture invert in 5% calf serum. They can be stabilized so that the epithelium has normal polarity by the addition of acid-soluble collagen to the medium. Only a small fraction of the cell surface is in contact with the collagen; in the presence of laminin the collagen fibers may not touch the cell surface during the stabilization. A basal lamina formed in the presence of laminin but in the absence of a collagen gel does not stabilize the polarity of the cells in 5% serum. Collagen is not the only agent that stabilizes since follicles are stabilized in a plasma clot (with L. Nitsch).

Involution of the hyperplastic thyroid (with O. Tachiwaki): Involution of the hyperplastic thyroid is a very different phenomenon from involution of

other tissues such as prostate gland or mammary gland. Rather than disintegrating, the epithelial cells persist with a well developed protein-synthetic apparatus. Although the epithelial cells become smaller in size there is no appreciable occurrence of autophagic vacuoles. There must be some pathway for the removal of cytoplasm and cytoplasmic organelles in addition to autophagy.

Proliferation of blood capillary endothelium in adipose tissue pads on the thyroid (with S. Smeds): Lipolysis in brown adipocytes during the feeding of thiourocil is accompanied by multiplication of capillary endothelial cells in the adipose tissue pads on the thyroid gland as indicated by tritiated thymidine labeling. The peak response is delayed relative to the response of thyroid capillaries or thyroid veins. The response appears to be due to elevated circulating thyrotropin, and is almost completely specific for adipose tissue at the thyroid. There is no response of adipose tissue at almost all other sites in the body.

Significance for Cancer Research and the Program of the Institute:

Enables us to understand more about the organization of cells including organization of the capillary bed, in normal and growing tissues.

Proposed Course: I plan to continue the above studies.

Publications:

Garbi, C., and Wollman, S.H.: Ultrastructure and some other properties of inverted thyroid follicles in suspension culture. Exp. Cell Res., 1982, in press.

Tachiwaki, O., and Wollman, S.H.: Shedding of dense cell fragments into the follicular lumen early in involution of the hyperplastic thyroid gland. Lab. Invest., 1982, in press.

Wollman, S.H., and Smeds, S.: Changes in thyroid blood vessels during the development of thyroid hyperplasia. In Andreoli, M., Monaco, F., and Robbins, J. (Eds.): Advances in Thyroid Neoplasia 1981. Rome, Field Educational Italia, 1981, pp. 1-10.

Zeligs, J., and Wollman, S.H.: Ultrastructure of cytokinesis in blood capillary endothelial cells in thyroid gland in vivo. J. Ultrastruct. Res. 75: 291-299, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08705-06 LMB
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PERIOD COVERED  
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)  
Genetic and Biochemical Analysis of Cell Behavior

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER  
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Michael M. Gottesman Chief, Molecular Cell Genetics Section LMB NCI

Others: Charles Roth Guest Worker LMB NCI  
Roberto Verna Guest Worker LMB NCI  
Irene Abraham Expert LMB NCI  
Paul Doherty Fogarty Fellow LMB NCI  
George Vlahakis Research Biologist LMB NCI  
Margaret Chapman Research Biologist LMB NCI  
Clark McClurkin Co-Step LMB NCI  
Mark Leitschuh Co-Step LMB NCI

COOPERATING UNITS (if any)

None

LAB/BRANCH

Laboratory of Molecular Biology

SECTION

Molecular Cell Genetics Section

INSTITUTE AND LOCATION

National Cancer Institute, Bethesda, Maryland 20205

TOTAL MANYEARS:

5.5

PROFESSIONAL:

5.5

OTHER:

0.0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We are utilizing the Chinese Hamster Ovary (CHO) fibroblast grown in vitro to study the genetics and biochemistry of some aspects of the behavior of cultured cells. Our work has emphasized morphology and its relationship to growth control, response to cyclic AMP, the role of cell surface antigens, receptors and internalization of their ligands in cell behavior, and the mechanism of cell transformation by tumor viruses. We have isolated a variety of different mutants with altered microtubules, abnormal morphology, or abnormal response to cyclic AMP and many of these have been characterized as to their biochemical defects. We have isolated cell mutants resistant to transformation by the product of the src gene of Rous sarcoma virus. We are establishing general procedures for the isolation of mutants unable to internalize specific ligands. These mutants are analyzed genetically using the techniques of somatic cell hybridization, gene cloning and gene transfer, and biochemically by classical enzymology, immunology, affinity labelling techniques and two-dimensional electrophoresis.

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

**Objectives:** To determine by genetic manipulation of CHO cells the cell proteins and processes needed to maintain the cytoskeleton, response to cAMP, transformation by Rous sarcoma virus, and internalization of ligands.

**Methods Employed:** Cell culture; virus infections; isolation of cell behavior mutants; karyotyping and G-binding; somatic cell hybridization; gene transfer using DNA and chromosomes; recombinant DNA techniques; immunologic techniques including immunoprecipitation, immunolocalization and use of monoclonal antibodies; one and two-dimensional electrophoretic and chromatographic analysis of DNA, RNA and proteins; enzymologic analyses.

**Major Findings:** (1) We have isolated multiple classes of mutants comprising more than 25 independent mutants which are resistant to the morphologic, transport, and growth inhibitory effects of increased levels of cyclic AMP. Eight of these independent mutants have been analyzed in detail. Each mutant has altered cAMP-dependent protein kinase activity indicating that these effects of cAMP in CHO fibroblasts are mediated through protein kinase. One of these mutants has a defect in the catalytic subunit of cAMP dependent protein kinase, and another has a defective Type I regulatory subunit. Some mutants lack Type II protein kinase and some lack Type I with the remaining enzyme in each case showing normal activity. This result indicates that both Type I and Type II cAMP-dependent protein kinases are essential for cAMP effects in CHO cells. Our continuing analysis of cAMP stimulated responses in cAMP<sup>R</sup> CHO cells demonstrates that increases in activity of the enzymes ornithine decarboxylase, cyclic nucleotide phosphodiesterase, and transglutaminase require an intact cAMP-dependent protein kinase system. In contrast, glycogen synthase activity in CHO cells does not appear to be regulated by levels of cAMP-dependent protein kinase activity.

(2) We have continued our analysis of cAMP-dependent phosphorylations in intact wild-type and mutant CHO cells and in cell lysates. The major cAMP-stimulated phosphorylation in intact cells involves a protein of  $M_r$  approx. 52,000. Other phosphoproteins are also substrates *in vivo* for cAMP-dependent protein kinase (e.g. intermediate filament protein) as detected by an alteration in their <sup>32</sup>P tryptic peptide fingerprints. When CHO cells are transformed by Rous sarcoma virus (RSV), the transforming function of the virus, pp60<sup>src</sup>, also becomes phosphorylated in intact cells in a cAMP-dependent manner.

(3) We have found that phosphorylation of pp60<sup>src</sup> has no apparent inhibitory effect on its function as a transforming protein in CHO cells. CHO-RSV cells have been found to be resistant to the growth inhibitory and morphological effects of cAMP despite the fact that the pp60<sup>src</sup> which they express is phosphorylated in a cAMP-dependent manner. Purified src gene product (pp54<sup>src</sup>)



can also be phosphorylated by pure catalytic subunit of cAMP-dependent protein kinase in vitro. The biochemical effects of this phosphorylation must await identification of physiological substrates for src.

(4) We have found that treatment of CHO cells with dibutyryl cAMP or cholera toxin results in a 3-5 fold increase in 8-azido-<sup>32</sup>P-cAMP photoaffinity labeling of free Type I regulatory subunit (RI) of cAMP-dependent protein kinase. This increase in RI is due to decreased degradation of RI to which cAMP or dbcAMP is bound. 8-Br-cAMP binding to RI does not stabilize it to degradation by cellular proteases. One of our cAMP<sup>R</sup> mutants (10248) with an altered RI is not stabilized by treatment with cholera toxin or dbcAMP. These results indicate that there are at least two non-identical cAMP binding sites on RI in CHO cells only one of which recognizes 8-Br-cAMP, and that occupation of the non-8-Br-cAMP site by cAMP results in stabilization of RI to intracellular degradation.

(5) We have begun to clone the gene coding for Type I regulatory subunit of cAMP-dependent kinase (RI). mRNA has been isolated from CHO cells. RI mRNA has been detected by immunoprecipitation or affinity chromatographic purification of RI from in vitro translations. A cDNA library has been prepared from CHO cells in pBR322 and a hybridization selection technique used to characterize E. coli clones carrying specific cDNAs. Several possible candidates for cDNAs coding for RI are currently being analyzed.

(6) We have isolated several independent CHO mutants resistant to the anti-mitotic drugs colcemid, griseofulvin and taxol. By two-dimensional electrophoretic analysis, mutants resistant to colcemid and griseofulvin have been shown to contain an altered beta-tubulin and one resistant to taxol has an altered alpha-tubulin. These proteins are the two major components of the microtubular system of the CHO cytoskeleton. These mutants are temperature-sensitive for growth, and their defect at the non-permissive temperature is due to a failure of normal cell division caused by a defect in spindle formation. These alpha and beta-tubulin mutants and their revertants have been analyzed using cloned alpha and beta-tubulin cDNAs and no evidence for gene rearrangement or deletion has so far been detected.

(7) As part of the development of techniques for the genetic analysis of CHO cells, we have successfully transformed these cells with DNA containing the cloned Herpes Simplex Virus thymidine kinase gene, a cloned E. coli xanthine-guanine phosphoribosyl transferase gene, and tk<sup>+</sup> CHO whole chromosomal DNA. Although the frequency of transformation of CHO cells is low, this frequency can be increased dramatically by cotransformation with an SV40 vector known as pSV2-gpt. These results suggest that it will be possible to use DNA transfer in linkage analysis of CHO mutations, to study gene regulation in mutant cell lines and as a means of direct cloning of genes by plasmid rescue techniques.

Significance for Cancer Research and the Program of the Institute:  
CHO cells will cause tumors in appropriate hosts. The identification of mutant CHO cells with specific defects in cell surface functions and in regulation of



cellular growth and morphology will enable us to determine whether any of these functions are needed for tumor formation. Once this information is obtained, specific therapy aimed at neutralizing those cell surface or cytoskeletal functions required for tumor formation can be devised.

### Objective 3, Approach 3.5

Proposed Course: To isolate many classes of mutants with abnormal cell behavior and determine specific protein alterations in these mutants. To determine the molecular basis for mutants already isolated and to use recombinant DNA technology to molecularly clone the components of the cAMP-dependent protein kinase system.

### Publications:

LeCam, A., Gottesman, M.M., and Pastan, I.: Glycogen synthetase activity in Chinese hamster ovary cells. Studies with wild-type and mutant cells defective in cyclic AMP-dependent protein kinase. Biochim. Biophys. ACTA 675: 94-100, 1981.

Gottesman, M.M., Singh, T., LeCam, A., Roth, C., Nicolas, J.C., Cabral, F., and Pastan, I.: Cyclic AMP-dependent phosphorylations in cultured fibroblasts: a genetic approach. Cold Spring Harbor Conferences on Cell Proliferation 8: 195-209, 1981.

Cabral, F., Abraham, I., and Gottesman, M.M.: Isolation of a taxol-resistant Chinese hamster ovary cell mutant with an alteration in  $\alpha$ -tubulin. Proc. Natl. Acad. Sci. USA 78: 4388-4391, 1981.

Abraham, I., Tyagi, J.S., and Gottesman, M.M.: Transfer of genes to Chinese hamster ovary cells by DNA-mediated transformation. Somatic Cell Genet. 8, 23-39, 1982.

Roth, C., Pastan, I., and Gottesman, M.M.: Rous sarcoma virus transformed cells are resistant to cyclic AMP. J. Cell Physiol. 111: 42-48, 1982.

Cabral, F., Abraham, I., and Gottesman, M.M.: Revertants of a CHO mutant with an altered  $\beta$ -tubulin: evidence that the altered tubulin confers both colcemid resistance and temperature sensitivity on the cell. Mol. Cell Biol., 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)		U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08706-11 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982			
TITLE OF PROJECT (80 characters or less) Mammary Carcinogenesis: MMTV - Cell Interaction			
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI:            Gilbert H. Smith            Research Biologist            LMB    NCI			
COOPERATING UNITS (if any) LCMB, DCCP, NCI Department of Cell Biology, Baylor University			
LAB/BRANCH Laboratory of Molecular Biology			
SECTION Molecular Cell Genetics Section			
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205			
TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 1.0	
CHECK APPROPRIATE BOX(ES)			
<input type="checkbox"/> (a) HUMAN SUBJECTS		<input type="checkbox"/> (b) HUMAN TISSUES	
		<input checked="" type="checkbox"/> (c) NEITHER	
<input type="checkbox"/> (a1) MINORS		<input type="checkbox"/> (a2) INTERVIEWS	
SUMMARY OF WORK (200 words or less - underline keywords) <u>Mammary tumorigenesis</u> induced by exogenous infection with MMTV is accompanied by <u>integration of viral DNA</u> into the transformed cell DNA. Acquisition of viral DNA by the transformed cell is thought to lead to malignant transformation. Mouse mammary tumors are also induced by genetically inherited MMTV proviral genes. We examined the DNA of 16 genetically induced C3H mouse mammary carcinomas for rearrangement or reintegration of MMTV proviral genes by restriction endonuclease digestion and Southern blot analysis. Amplification of endogenous MMTV DNA in these tumors by reintegration was rare. Despite this all the tumors were expressing viral genes as measured by <u>radioimmune competition assay</u> , <u>immunoperoxidase</u> and <u>electron microscopy</u> . Examination of tumor DNA after digestion with methy-sensitive HpaII restriction endonuclease showed that proviral DNA was hypomethylated, i.e. associated with transcriptionally active DNA. Therefore, mammary tumorigenesis by MMTV may occur in the absence of MMTV DNA reintegration and transformation of mammary epithelium may instead be mediated through direct expression of viral gene products or alternatively by the expression of a virus gene product-induced cellular gene.			

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: The aim of the project is to elucidate the cell-virus-provirus interactions in the mouse mammary gland leading to epithelial cell hyperplasia and eventually to neoplasia. Multiple factors, including virus, chemicals, hormonal stimulation of the gland and genetic susceptibility, have been identified as playing important roles in the development of mammary cancer. Therefore our approach includes a multidisciplinary analysis of the intracellular events associated with mammary gland differentiation and development and the role of mammary tumor virus and chemical carcinogens in these normal events and those accompanying preneoplasia and neoplasia in the mammary epithelial tissue. We have initiated investigations into the role of chemical carcinogens in mammary cancer to determine whether they operate synergistically or independently of the tumor virus and whether chemicals have different targets within the tissue than the virus. To this end we are evaluating a new mouse model for experimental breast cancer, which gives promise of greater relevancy to the human condition. Special emphasis will be given to the physiological state of the epithelial tissue in relation to its response to the carcinogenic agent.

Major Findings: MMTV-mediated mammary tumorigenesis is accompanied by integration of viral DNA into the transformed cell DNA. Acquisition of viral DNA by the transformed cell is thought to lead to malignant transformation. In support of this, MMTV-induced preneoplastic lesions and tumors arising from these preneoplastic populations have been shown to be clonal or quasi-clonal with respect to the genomic position of the newly acquired MMTV DNA. Mouse mammary tumors are also induced by genetically inherited MMTV proviral genes. We examined the DNA of 16 genetically induced C3H mouse mammary carcinomas for rearrangement or reintegration of MMTV proviral genes by restriction endonuclease digestion and Southern blot analysis. We found that amplification of endogenous MMTV DNA in these tumors by reintegration was rare. Despite this all the tumors were expressing viral genes as measured by radioimmune competition assay, immunoperoxidase and electron microscopy. In agreement with this evidence for MMTV proviral expression, examination of tumor DNA after digestion with methyl-sensitive HpaII restriction endonuclease showed that the proviral DNA was hypomethylated, i.e. associated with transcriptionally active DNA. These studies demonstrate that mammary tumorigenesis by MMTV may occur in the absence of MMTV DNA reintegration and suggest that transformation of mammary epithelium may instead be mediated through direct expression of viral gene products or alternatively by the expression of a virus gene product-induced cellular gene.

The hormonal regulation of gene expression in tumors and explant cultures from mammary tissues has been studied using molecular hybridization, Northern and Southern blot analyses, in situ hybridization, electron microscopy and

radioimmunological techniques. Our studies to date have shown that regulation of casein and  $\alpha$ -lactalbumin gene expression are disjunctive in early preneoplastic lesions as determined by analyses of casein and  $\alpha$ -lactalbumin synthesis after hormonal stimulation in explant culture. The expression of proviral MMTV and casein genes was constitutive in the transformed cells as measured by molecular hybridization. Comparison of the regulation and expression of MMTV proviral and cellular genes in preneoplastic lesions, tumors arising from these lesions, and normal mammary glands may lead to the identification of cellular gene products important to the maintenance of the transformed phenotype in mammary dysplasia.

Our laboratory has developed a method for molecular and immunological identification of macromolecular components at the light and electron microscopic level in epoxy-resin embedded tissues. It is expected that this method will contribute significantly to our ability to localize and identify specific cellular gene activity.

Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 3, Approach 3. The mouse mammary tumor virus problem is a relevant experimental model for understanding human mammary neoplasia. The project is designed to provide a scientific basis to further our understanding of intracellular events during normal development of the mammary gland as well as during malignant transformation.

Proposed Course: To specify the tumorigenic influence of the mammary tumor virus, its relationship to normal differentiation and to the cellular response during chemical carcinogenesis, further to clarify the regulatory events responsible for the expression of endogenous retrovirus genes and their role in mammary tumorigenesis.

Publications:

Smith, G.H., and Vonderhaar, B.K.: Functional differentiation in mouse mammary gland epithelium is attained through DNA synthesis, inconsequent of mitosis. Dev. Biol. 88: 167-179, 1981.

Vonderhaar, B.K., and Smith, G.H.: Dissociation of cytological and functional differentiation in virgin mouse mammary gland during DNA synthesis inhibition. J. Cell Sci. 53: 97-114, 1981.

Medina, D., Socher, S.H., Smith, G.H., Dusing-Swartz, S., Arthur, L.O., and Butel, J.S.: Separate pathways for viral and chemical carcinogenesis in the mouse mammary gland. In Rich, M., and Furmanski, P. (Eds.): Biological Carcinogenesis. Marcel Dekker, Inc., New York, 1981, in press.

Smith, G.H., and Vlahakis, G.: Separation of high mammary tumor incidence from high hepatoma incidence in backcross mice during segregation of the viable yellow gene. Int. J. Cancer, 1982, in press.

Smith, G.H., Henry, T.J., Vlahakis, G., and Arthur, L.O.: Suppression of spontaneous mammary tumorigenesis despite Mtv-1 gene expression in hybrid and backcross C3H-AVYfb x C3H/Sm mice. Int. J. Cancer, 1982, in press.

Drohan, W.N., Benade, L.E., Graham, D.E., and Smith, G.H.: MMTV proviral sequences congenital to C3H/Sm mice are differentially hypomethylated in chemically-induced, virus-induced and spontaneous mammary tumors. J. Virol., 1982, in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08707-09 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less) Relationship of Genetic and Non-Genetic Factors in Mouse Mammary Tumorigenesis		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	George Vlahakis	Research Biologist LMB NCI
Other:	Bernard Sass	Research Pathologist CGT NCI
COOPERATING UNITS (if any)  Carcinogenesis Testing Program, DCCP, NCI		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Molecular Cell Genetics Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.25	PROFESSIONAL: 0.25	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Collaborative work continues into the relationship of <u>genetic</u> and <u>non-genetic factors</u> in <u>spontaneous mouse tumorigenesis</u> . Interest is in the different lines of naturally occurring <u>mammary tumor virus</u> (MTV), and in the genetically variable <u>inbred mouse</u> strains. Interest also is in the manner of <u>transmission</u> of the various MTV's, i.e., whether by female or equally well by either parent. Finally, strains of mice that develop <u>hormone responsive</u> mammary gland tumors and <u>hyperplastic alveolar nodules</u> from which malignant mammary tumors arise are also of interest for <u>histogenesis</u> studies.		

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: To study the relationship of genetic and non-genetic factors in mouse mammary tumorigenesis.

Findings and Results: Collaborative studies with other investigators are continuing again this year.

A manuscript with Gerald L. Princler and K. Robert McIntire of the Laboratory of Immunodiagnosis, which deals with the dynamics of serum alpha-fetoprotein in the high spontaneous hepatoma mouse strain C3H-AVYfB, has now been published in the European Journal of Cancer and Clinical Oncology.

A paper with Bernard Sass of the Registry of Experimental Cancer, Division of Cancer Cause and Prevention, dealing with the histogenesis and biology of mammary gland lesions occurring spontaneously in female mice has been submitted for publication in Toxicological Pathology. One of the precursor lesions we studied was the hyperplastic alveolar nodule from which most mammary tumors arise in females carrying mammary tumor virus (MTV). Another precursor mammary gland lesion of interest to us was the plaque which occurs in several of the high spontaneous mammary tumor mouse strains not native to the United States. We were also interested in the pregnancy dependent mammary tumors observed in the European strain GR. These tumors normally regress at parturition but reappear in subsequent pregnancies and eventually become malignant. One morphologically distinct mammary tumor in GR classified as pale cell carcinoma is of particular interest. This tumor apparently is associated with the line of MTV present in strain GR.

Two manuscripts with Gilbert Smith in our laboratory have now been revised for publication in the International Journal of Cancer. They deal with the biology, virology and biochemistry of spontaneous mammary gland tumors in hybrid and backcross females made with the high mammary tumor and hepatoma strain C3H-AVYfB and the low tumor strain C3H/Sm.

Significance to Cancer Research and the Program of the Institute:  
Objective 1, Approach 4.

The occurrence of spontaneous mammary gland tumors in laboratory mouse strains depends on the relationship of several factors: host genotype, mammary tumor virus, hormones, environment. Mouse mammary tumorigenesis with the variable tumor strains needs to be continued so that eventually mechanisms of action between the various factors will be understood and might, therefore, provide better insight into the disease process in humans.

Publications

Reuber, Melvin D., Vlahakis, G. and Heston, W.E.: Spontaneous hyperplastic and neoplastic lesions of the uterus in mice. J. Gerontol. 36: 663-673, 1981.

Princler, G.L., Vlahakis, G., Kortright, K.H., Okada, S. and McIntire, K.R.: Dynamics of serum alpha-fetoprotein during spontaneous hepatocellular carcinoma development in mice. Eur. J. Cancer Clin. Oncol. 17: 1241-1248, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08709-07 LMB											
PERIOD COVERED October 1, 1981 through September 30, 1982													
TITLE OF PROJECT (80 characters or less)  NAD Metabolism and ADPriboseylation of Proteins													
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT													
<table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 40%;">George S. Johnson</td> <td style="width: 25%;">Research Chemist</td> <td style="width: 20%;">LMB NCI</td> </tr> <tr> <td rowspan="2">Others:</td> <td>Narimichi Kimura</td> <td>Visiting Fellow</td> <td>LMB NCI</td> </tr> <tr> <td>Nobuko Kimura</td> <td>Visiting Associate</td> <td>LMB NCI</td> </tr> </table>			PI:	George S. Johnson	Research Chemist	LMB NCI	Others:	Narimichi Kimura	Visiting Fellow	LMB NCI	Nobuko Kimura	Visiting Associate	LMB NCI
PI:	George S. Johnson	Research Chemist	LMB NCI										
Others:	Narimichi Kimura	Visiting Fellow	LMB NCI										
	Nobuko Kimura	Visiting Associate	LMB NCI										
COOPERATING UNITS (if any)  None													
LAB/BRANCH Laboratory of Molecular Biology													
SECTION Molecular Cell Genetics Section													
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205													
TOTAL MANYEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0.0											
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS													
SUMMARY OF WORK (200 words or less - underline keywords)													
<p>Membrane associated <u>GDP kinase</u> activity increases in cultured normal rat kidney cells treated with <u>2-pyridine carboxylic acid</u>. This increase is associated with an augmented <u>agonist activation of adenylate cyclase</u>. Known components of adenylate cyclase are unaffected by drug treatment. Results supply experimental evidence for the proposal that GDP kinase is an essential component in regulation of adenylate cyclase activity.</p> <p>Syntheses of <u>growth hormone</u> and <u>prolactin</u> by cultured rat <u>pituitary GH3</u> cells are increased by <u>treatment of cells with inhibitors of ADPriboseylation</u>. Growth hormone synthesis is increased synergistically by treatment with both ADPriboseylation inhibitors and <u>thyroid hormone</u>, a physiological stimulator of growth hormone synthesis. <u>mRNA</u> contents increase in parallel with the increases in protein syntheses. Results point to a role for ADPriboseylation in regulation of <u>gene transcription</u>.</p>													

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

#### PROJECT DESCRIPTION:

Objectives: Nicotinamide adenine dinucleotide (NAD) is known to undergo two major types of reactions in mammalian cells, reduction to NADH and cleavage at the nicotinamide-ribose linkage to form nicotinamide and adenosine diphosphate ribose. ADPribose can be covalently attached to proteins or it can polymerize to form poly (ADPribose). NAD is present in the cell at a concentration of about 0.1-0.3 mM, and since the half time for degradation of total cellular NAD at this cleavage site is just 1-2 hours, this reaction is a major cellular activity. Despite an extensive research effort from numerous laboratories, the biological purpose for this NAD cleavage reaction remains obscure.

The object of this project is to understand the regulation of NAD metabolism and to discover and evaluate the physiological functions for ADPriboseylation of proteins.

Methods Employed: Standard culture techniques will be used to grow cells. Thin layer chromatography, high performance liquid chromatography, and two dimensional gel electrophoresis will be used to analyze metabolic components and proteins. Standard biochemical techniques will be used to measure cyclic AMP, NAD, adenylate cyclase activity, RNA metabolism, and protein synthesis. mRNA will be detected and quantitated by hybridization to specific cDNA sequences.

#### Major Findings:

Cyclic AMP Metabolism: Previously we observed that treatment of cultured normal rat kidney (NRK) cells with 2-pyridine carboxylic acid resulted in an increase in hormone-stimulated adenylate cyclase activity. During the past year, this complex enzyme has been analyzed in detail in an attempt to understand the molecular basis for this increase in activity. None of the known components of adenylate cyclase are altered by treatment of cells with 2-pyridine carboxylic acid, but interestingly, an additional enzyme activity, membrane associated GDP kinase, is increased by the treatment. This activity has been proposed, but not directly demonstrated, to be a component of the adenylate cyclase complex. Our observation provides some experimental basis for this proposal and provides the framework for future studies designed to understand regulation of adenylate cyclase activity.

Gene Regulation: Cultured rat pituitary tumor cells (GH<sub>3</sub> cell line) synthesize growth hormone (GH) and prolactin (PRL). When the cells are treated with agents which inhibit ADPriboseylation, syntheses of these two proteins are increased; syntheses of other cellular proteins are unaffected. Thyroid hormone is a physiological stimulator of GH synthesis. GH synthesis is



increased synergistically when GH<sub>3</sub> cells are treated with both thyroid hormone and ADPriboseylation inhibitors. GH and PRL mRNAs increase in parallel with the increases in protein syntheses under the various treatments. The results of this study point to a role for ADPriboseylation in regulation of gene transcription.

Significance for Cancer Research and the Program of the Institute: By understanding how growth and metabolism are regulated and by understanding differences in this regulation between normal and transformed cells we may learn to control the growth of certain cancer cells.

Proposed Course: GDP kinase will be purified and antibodies against it raised. The effects of the pure enzyme and its antibody on adenylate cyclase activity in intact membranes and in solubilized-reconstituted membranes will be tested. These studies will be used to demonstrate more directly whether or not GDP kinase is a component of adenylate cyclase.

We will attempt to determine the biochemical basis for the effects of ADPriboseylation inhibitors on gene expression. To begin this study three approaches will be taken: 1. Proteins which are ADPriboseylated in isolated nuclei and in intact cells will be analyzed by one and two dimensional gel electrophoresis. The effect of thyroid hormone treatment on these ADPriboseylations will be determined. 2. Thyroid hormone binding to chromatin from cells treated with ADPriboseylation inhibitors and the possibility that proteins which influence its binding to chromatin are ADPriboseylated will be explored. 3. Attempts will be made to synthesize GH mRNA in nuclei isolated from control cells or cells treated with ADPriboseylation inhibitors. The effect of NAD on this nuclear RNA synthesis will be tested.

Publications:

Costantini, M.G., and Johnson, G.S.: Disproportionate accumulation of 18S and 28S ribosomal RNA in cultured normal rat kidney cells treated with picolinic acid or 5-methylnicotinamide. Exp. Cell Res. 132: 443-451, 1981.

Johnson, G.S., and Chiang, P.K.: 1-methylnicotinamide and NAD metabolism in normal and transformed normal rat kidney cells. Arch. Biochem. Biophys. 210: 263-269, 1981.

Johnson, G.S.: Benzamide and its derivatives inhibit nicotinamide methylation as well as ADPriboseylation. Biochem. Int. 2: 611-617, 1981.

Johnson, G.S.: 1-methylnicotinamide metabolism in cultured cells. In Usdin, E., Borchardt, R., and Creveling, C. (Eds.): Biochemistry of S-adenosylmethionine and Related Compounds. McMillan Press, New York, 1982, pp. 513-520.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08710-07 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less)  <u>DNA Replication In Vitro</u>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Sue Wickner, Research Chemist                      LMB NCI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Biochemical Genetics Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.8	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <p style="margin-left: 40px;">             The molecular mechanisms involved in <u>DNA replication</u> are being studied. Proteins involved in both <u>phage lambda</u> and <u>E. coli</u> replication are being purified and studied <u>in vitro</u> for their interactions with each other and with DNA. Attempts are being made to reproduce in vitro, the in vivo pathway of replication of lambda DNA in extracts of phage infected bacteria supplemented with phage replication proteins. The present emphasis is to gain insight into the process of initiation of chromosome replication.           </p>		

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objective: To gain information about basic biochemical mechanisms involved in the process of DNA replication.

Methods Employed: Biochemical techniques required for protein purification, enzyme assays, phage and plasmid DNA preparation, and nucleic acid sequencing.

Major Findings: (1) Initiation of phage lambda DNA replication: Lambda replication in vivo requires two phage proteins, the O and P gene products, in addition to host proteins including dnaB, polC, dnaG, dnaN, dnaZ, dnaJ, dnaK, grpD, and grpE gene products and RNA polymerase. In collaboration with K. Zahn in F. Blattner's laboratory, we purified the lambda O protein from cells carrying a plasmid on which the O gene was cloned. The purified protein binds specifically to DNA containing the lambda origin of replication. In addition, the O protein also binds to the purified lambda P protein. No enzymatic activities have been found associated with either one of these proteins yet. We are further characterizing these proteins individually, together, and with lambda DNA to understand their role in the initiation of replication. We are also using these proteins in conjunction with crude E. coli proteins and lambda DNA in attempts to reconstruct  $\lambda$  replication in vitro.

(2) E. coli DNA replication: In collaboration with J. Walker, several new E. coli mutants blocked in DNA replication are being studied in vitro. Preliminary evidence suggests one of these is defective in a subunit of DNA polymerase III.

Significance for Cancer Research and the Program of the Institute: The central process of heredity and cell growth is the replication of the genetic material. Studies of this process in E. coli have been made possible by the availability of (1) mutants defective in DNA synthesis and (2) large amounts of bacteria necessary for biochemical studies. It is expected that the understanding that this work is generating about the process of DNA replication in E. coli will shed light on the nature of the same process in animal cells. Perhaps if normal replication were understood, we would be better able to study the abnormalities of cancer cells.

Proposed Course: To continue studying biochemical mechanisms involved in DNA synthesis using purified proteins and defined DNA templates.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08711-06 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Mechanism of Site-Specific Recombination in Bacteriophage Lambda		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Sue Wickner, Research Chemist LMB NCI  Others: Susan Gottesman, Research Chemist LMB NCI Jeffrey Auerbach, Guest Worker LMB NCI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Biochemical Genetics Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  We have been studying the biochemistry and genetics of <u>lambda site-specific recombination</u> . We have purified the proteins involved in <u>excisive site-specific recombination</u> of bacteriophage lambda. We are studying the role of the phage-coded functions, <u>Xis</u> and <u>Int</u> , and host-coded functions in this recombination reaction. We are extending these studies to the <u>in vitro</u> recombination reaction. We are isolating mutations in <u>Int</u> and <u>Xis</u> which alter the interaction of these proteins during integration and excisive recombination.		

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objective: Bacteriophage lambda inserts into and excises from the E. coli chromosome. This recombination occurs at a unique site in the viral and host chromosomes, and is promoted by several functions. We have previously demonstrated the excision reaction in vitro. Our object is to continue to study the mechanism of phage excision, by purifying and analyzing the components of the in vitro system, and to understand how the excision reaction differs from the forward, integration reaction at a mechanistic level.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) We have developed methods for extensively purifying the protein components required for lambda excision, Int, Xis, and host factors. Using these purified proteins, we are studying the mechanism of the recombination reaction. We have shown that the same host factors that are required for lambda integration are also required for excision. We have also shown that Xis, in the absence of Int and host factors, binds to DNA containing sites for excision. In collaboration with Bruce Howard, who is constructing a vector system for shuttling DNA between bacterial and mammalian cells, we are developing in vitro conditions for excising DNA flanked by lambda prophage attachment sites from mammalian chromosomal DNA.

(2) We have devised genetic selections for isolating lambda phage mutants that undergo site specific recombination in E. coli strains lacking the active host factors normally required for this reaction. We have genetically mapped and characterized these mutants as to their host factor requirement.

Significance for Cancer Research and the Programs of the Institute: Integration and excision of the bacterial virus lambda may serve as a model for understanding the integration and excision mechanisms of animal viruses.

Proposed Course: We will continue characterizing the lambda exciseive recombination reaction in vitro focusing on protein-protein interactions and DNA-protein interactions. We will also continue the characterization of phage and host mutants both in vivo and in vitro.

Publications:

Abremski, K., and Gottesman, S.: Purification of the bacteriophage lambda Xis base product required for  $\lambda$  exciseive recombination. J. Biol. Chem., 1982, in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08712-07 LMB																				
PERIOD COVERED October 1, 1981 through September 30, 1982																						
TITLE OF PROJECT (80 characters or less)  The Role of Plasma Membrane Proteins in the Regulation of Cell Behavior																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																						
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 70%;">Ira Pastan, Chief, Laboratory of Molecular Biology</td> <td style="width: 10%;">LMB</td> <td style="width: 10%;">NCI</td> </tr> <tr> <td></td> <td>Mark Willingham, Chief, Ultrastructural Cytochemistry Section</td> <td>LMB</td> <td>NCI</td> </tr> <tr> <td>Others:</td> <td>Robert Dickson, Guest Worker</td> <td>LMB</td> <td>NCI</td> </tr> <tr> <td></td> <td>Richard Schlegel, Assistant Senior Surgeon</td> <td>LMB</td> <td>NCI</td> </tr> <tr> <td></td> <td>John Hanover, Postdoctoral Fellow</td> <td>LMB</td> <td>NCI</td> </tr> </table>			PI:	Ira Pastan, Chief, Laboratory of Molecular Biology	LMB	NCI		Mark Willingham, Chief, Ultrastructural Cytochemistry Section	LMB	NCI	Others:	Robert Dickson, Guest Worker	LMB	NCI		Richard Schlegel, Assistant Senior Surgeon	LMB	NCI		John Hanover, Postdoctoral Fellow	LMB	NCI
PI:	Ira Pastan, Chief, Laboratory of Molecular Biology	LMB	NCI																			
	Mark Willingham, Chief, Ultrastructural Cytochemistry Section	LMB	NCI																			
Others:	Robert Dickson, Guest Worker	LMB	NCI																			
	Richard Schlegel, Assistant Senior Surgeon	LMB	NCI																			
	John Hanover, Postdoctoral Fellow	LMB	NCI																			
COOPERATING UNITS (if any) Elizabeth Neufeld, NIAMDD L. Charles Smith, Department of Medicine, Baylor College of Medicine and the Methodist Hospital, Houston, Texas 77030 V. Macchia, Institute of General Pathology, University of Naples, Naples, Italy																						
LAB/BRANCH Laboratory of Molecular Biology																						
SECTION Office of the Chief																						
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205																						
TOTAL MANYEARS: 4.4	PROFESSIONAL: 3.4	OTHER: 1.0																				
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																						
SUMMARY OF WORK (200 words or less - underline keywords)  <p>We have identified a new organelle, the <u>receptosome</u> that carries ligands from the <u>clathrin</u> coated pits on the surface of <u>fibroblasts</u> to the <u>Golgi</u> apparatus. Ligands carried by this pathway include <u><math>\alpha</math>2-macroglobulin</u>, <u>epidermal growth factor</u>, <u>insulin</u>, <u>low density lipoprotein</u>, <u><math>\beta</math>-galactosidase</u> and some <u>viruses</u>. Ligands that eventually end up in lysosomes first tranverse the reticular portion of the Golgi and are concentrated in coated pits of the Golgi before they enter lysosomes. We believe that receptosomes form directly from the coated pits of the cell surface and not by uncoating of coated vesicles.</p>																						

PHS-NIH  
Individual Project Report  
October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: To identify the membrane proteins that participate in cell adhesion, cell movement and regulate cell metabolism and growth.

Methods: Cell culture; preparation and analysis of membrane proteins and their effects on cell behavior; isolation of mutants with defective membrane proteins.

Major Findings: With M. Willingham and coworkers we have identified a new organelle, the receptosome, that carries ligands from coated pits in the plasma membrane to the cell interior. The receptosome prevents ligands from being directly transferred to lysosomes and instead transports them to the Golgi from which some ligands go on to lysosomes. Ligands that traverse this pathway are  $\alpha_2$ -macroglobulin ( $\alpha_2$ M), insulin, epidermal growth factor, triiodothyronine, low density lipoprotein (with D. Via, A. Gotto and C. Smith),  $\beta$ -galactosidase (with G. Sahagian and E. Neufeld). In addition some viruses including vesicular stomatitis virus (VSV) and adenovirus use the coated pit receptosome pathway.

Dansylcadaverine (dc) has been found to slow the entry of many soluble ligands. R. Schlegel has now found that dc inhibits the entry of VSV. He has also found rimantadine and amantadine, two antiviral agents, inhibit the entry of  $\alpha_2$ M. These findings indicate that the steps involved in the entry of VSV and  $\alpha_2$ M via coated pits probably have a common enzymatic basis.

It has been widely believed that during receptor-mediated endocytosis the large coated pits (~400 Å diameter) pinch off to form coated vesicles which subsequently lose their clathrin coat and the clathrin recycles to the cell surface to form new coated pits. Recent experiments have suggested a different mechanism in which coated pits remain permanently attached to the cell surface and uncoated vesicles (receptosomes) form directly from the coated pits. One set of morphologic experiments by M. Willingham has shown that at 1°C, all coated pits are in direct communication with the cell surface, some by long tortuous necks. The second set of experiments by J. Wehland has shown that microinjection of antibodies to clathrin into living cells fails to precipitate clathrin (as would be expected if the molecule becomes free of the coated vesicle membrane during recycling to the cell surface) or to interfere with receptor-mediated endocytosis.

R. Schlegel and R. Dickson have found that various monovalent ionophores also inhibit VSV and  $\alpha_2$ M entry. These compounds do not interfere with the entry of ligands into coated pits but they slow their transfer to receptosomes. These findings are consistent with a model in which receptosomes form from coated pits and the force forming the receptosome is osmotic pressure generated by an ion pump.

Previous studies have shown that  $\alpha_2$ M, EGF and  $\beta$ -galactosidase traverse the Golgi region on the way to lysosomes. It has now been possible to determine which portion of the Golgi is involved. When KB cells are allowed to take up EGF coupled to horseradish peroxidase (EGF-HRP), the ligand appears in the reticular portion of the Golgi (but not the Golgi stacks) 10-12 min after entry. The ligand is next found clustered in the small clathrin-coated pits (~800 Å diameter) which are found on this portion of the Golgi. A few minutes later EGF begins to appear in lysosomes. These data suggest that the coated pits of the Golgi and those at the cell membrane act in an analogous manner as concentrating organelles. The latter concentrates molecules on the way out of the Golgi and sends them on to lysosomes; the former concentrates ligands at the cell surface prior to cellular entry.

Significance to Biomedical Research and the Program of the Institute:  
Objective 3, Approach 3.5.

The invasive properties of tumor cells may be due to altered membrane proteins. Identification and isolation of the proteins may suggest new methods of cancer treatment.

Proposed Course: Establish the biochemical basis of how receptor-ligand complexes cluster in pits and how receptosomes form.

Publications:

Pastan, I.H., and Willingham, M.C.: The internalization of insulin and other hormones by fibroblastic cells. Festschrift Symposium Honoring Dr. Rachmiel Levine, Eisenhower Medical Center, Rancho Mirage, California. Diabetes Care 4: 33-37, 1981.

Pastan, I., Haigler, H., Dickson, R., Cheng, S.-y., and Willingham, M.: The Role of the Receptosome in Receptor-Mediated Endocytosis. Miami Winter Symposia Series. In Mozes, L., Schultz, J., Scott, W.A., and Werner, R. (Eds.): Cellular Responses to Molecular Modulators. New York, Academic Press, 1981, Vol. 18, pp. 137-148.

Dickson, R.B., Nicolas, J-C., Willingham, M.C., and Pastan, I.: Internalization of  $\alpha_2$ -macroglobulin in receptosomes: Studies with monovalent electron microscopic markers. Exp. Cell Res. 132: 488-493, 1981.

Macchia, V., Caputo, G., Mandato, E., Rocino, A., Adhya, S., and Pastan, I.: Guanylate cyclase activity in Escherichia coli defective in adenylate cyclase. J. Bact. 147: 931-934, 1981.

Dickson, R.B., Willingham, M.C., Gallo, M., and Pastan, I.: Inhibition by bacitracin of high affinity binding of  $^{125}$ I- $\alpha_2$ M to plasma membranes. FEBS Letters 126: 265-268, 1981.

Pastan, I., and Willingham, M.: Journey to the center of the cell: role of the receptosome. Science 214: 504-509, 1981.

Wehland, J., Willingham, M.C., Dickson, R., and Pastan, I.: Microinjection of anti-clathrin antibodies into fibroblasts does not interfere with the receptor-mediated endocytosis of  $\alpha_2$ -macroglobulin. Cell, 25: 105-120, 1981.

Willingham, M.C., Rutherford, A.V., Gallo, M.C., Wehland, J., Dickson, R.B., Schlegel, R., Pastan, I.H.: Receptor-mediated endocytosis in cultured fibroblasts: Cryptic coated pits and the formation of receptosomes. J. Histochem. Cytochem. 29: 1003-1013, 1981.

Willingham, M., Pastan, I., Sahagian, G., Jourdian, G., and Neufeld, E.: A morphologic demonstration of the pathway of internalization of a lysosomal enzyme through the mannose 6-phosphate receptor in cultured CHO cells. Proc. Natl. Acad. Sci. USA 78: 6967-6971, 1981.

Dickson, R.B., Schlegel, R., Willingham, M.C., and Pastan, I.: Binding and internalization of  $\alpha_2$ -macroglobulin by cultured fibroblasts: Effects of monovalent ionophores. Exp. Cell Res., in press.

Via, D.P., Willingham, M.C., Pastan, I., Gotto, A.M., Jr., and Smith, L.C.: Co-clustering and internalization of low density lipoproteins and  $\alpha_2$ -macroglobulin in human skin fibroblasts. Exp. Cell Res., in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08714-05 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Mode of Action of a Bacterial Function Involved in Cell Growth Control		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Susan Gottesman, Research Chemist LMB NCI  Others: Patsy Trisler, Research Biologist LMB NCI Saeko Mizusawa, Visiting Fellow LMB NCI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Biochemical Genetics Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.6	PROFESSIONAL: 2.6	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  The lon mutants of <u>E. coli</u> have pleiotropic effects on cell growth: (a) they are defective in the formation of septa between dividing cells after treatment with ultraviolet light and therefore form long filaments, (b) they overproduce the mucopolysaccharide coating of cells, possibly disrupting the control of operons involved in synthesis of mucopolysaccharide, such as the <u>gal</u> operon, (c) they are defective in the <u>degradation of abnormal proteins</u> . We are investigating the genetic defect in the <u>lon</u> mutants with the aim of determining the interrelationships of these defects. We have found that the product of an <u>E. coli</u> gene, <u>suIA</u> , which seems to negatively regulate <u>cell division</u> , is highly unstable in <u>lon</u> <sup>+</sup> cells but is stable in <u>lon</u> <sup>-</sup> cells. This result suggests that the degradation of natural <u>E. coli</u> proteins may play an important role in regulating cell growth.		



PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: Lon mutants are mucoid, UV sensitive, and defective in degradation of abnormal proteins. We are investigating the genetics of these mutations as a basis for future biochemical characterization to understand the role of protein degradation in regulating cell growth.

Methods Employed: Standard microbial genetic and biochemical techniques.

Major Findings: (1) Using operon fusions of the lacZ gene to promoters involved in capsular polysaccharide synthesis, we have isolated secondary mutations which affect polysaccharide synthesis. One of these mutations has been mapped near minute 23 of the E. coli map, and is being characterized further. The interaction of lon control of these operons and control by the new regulatory mutation has been investigated; the mutations seem to have independent effects.

(2) The E. coli sulA gene has been cloned onto a  $\lambda$  vector and the product of the gene identified as a 17,000 dalton polypeptide. Genetic analysis suggests strongly that this protein is an inhibitor of cell septation, made after DNA damage. The sulA protein is highly unstable, with a half-life of about 1 minute. In lon<sup>-</sup> cells, however, the half-life is extended to about 19 minutes. This difference in half-life is sufficient to explain the abnormally long inhibition of septation which occurs in lon strains after DNA damage, and suggests that the degradation of critical E. coli proteins may play an important role in regulating cell growth. .

(3) The E. coli lon gene has been cloned onto a  $\lambda$  vector and a complementation assay for lon function developed.

Significance for Cancer Research and the Program of the Institute: An understanding of the growth control of E. coli may serve as a model for understanding growth control in normal and transformed mammalian cells. Protein degradation is believed to play an important role in the control of mammalian cells; insight into such processes should be gained by further investigation of the process in E. coli.

Proposed Course: Identification of mechanism of control of genes affected by lon, via genetic analysis and in vitro studies of lac fusions; identification of product through which lon effect on polysaccharide synthesis is mediated and analysis of its role in the physiology of the cell; identification of sulA product and its mechanism of regulation by lon.

Publications:

Gottesman, S., Halpern, E., and Trisler, P.: Role of sulA and sulB in filamentation by lon mutants of Escherichia coli K-12. J. Bacteriol. 148: 265-273, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08715-04 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less) Control of Synthesis of a Transformation-Dependent Secreted Glycoprotein		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Michael M. Gottesman Chief, Molecular Cell Genetics Section LMB NCI  Others: George Vlahakis Research Biologist LMB NCI Margaret Chapman Research Biologist LMB NCI Ling Hua Fogarty Fellow LMB NCI Paul Doherty Fogarty Fellow LMB NCI Gary Sahagian Staff Fellow IRP NIAMDD		
COOPERATING UNITS (if any) Laboratory of Experimental Pathology, DCCP, NCI		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Molecular Cell Genetics Section		
INSTITUTE AND LOCATION National Cancer Institute, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.0	PROFESSIONAL: 2.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Cultured <u>mouse fibroblasts</u> which are transformed by RNA viruses, a DNA virus or a chemical agent, all secrete a 35,000 Mr-glycoprotein ( <u>major excreted protein, MEP</u> ) in large amounts. Nontransformed murine fibroblasts secrete this protein in much lower amounts. These fibroblasts and cultured primary mouse epidermal cells, the target for tumor promoters <u>in vivo</u> , can be stimulated to release MEP by treatment with tumor promoters. We have purified this protein, prepared monospecific antisera against it, and begun to clone a cDNA which codes for MEP. The protein, of unknown biologic function, undergoes extensive modification prior to secretion. One of these modifications is the addition of mannose 6-phosphate, the lysosomal recognition marker. In non-transformed cells from which MEP is not secreted, MEP appears to have a predominantly lysosomal localization. We are studying this system as a model of <u>regulation of protein synthesis, processing and secretion</u> as it is affected by transformation and agents which mimic the transformed state, such as <u>tumor promoters</u> .		

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: To determine the mechanism of the control of synthesis, processing, and secretion of the major secreted protein of murine fibroblasts.

Methods Employed: Cell culture; radiolabeling of cell proteins, electrophoretic and chromatographic techniques; immunoprecipitation; in vitro translation of mRNAs; recombinant DNA technology.

Major Findings: (1) We have identified the major excreted protein (MEP) of transformed murine cells. Synthesis and secretion of MEP is increased in all transformed murine fibroblasts and is well-correlated with anchorage independence, and hence with tumorigenicity. Rat and hamster cell lines synthesize a similar protein which cross-reacts with MEP. Synthesis of MEP is stimulated by treatment of cells with tumor promoters and growth factors, such as PDGF.

(2) The purified MEP is extremely heterogeneous, with variation in molecular weight and charge. The molecular weight variation may be accounted for by variable glycosylation. The charge variation is due to the presence of multiple mRNA species for MEP, or variations in translation of a single species. At least 1 in 5 mannose residues on MEP are phosphorylated.

(3) The presence of mannose 6-phosphate on MEP enables it to bind quantitatively to the lysosomal phosphomannosyl receptor. MEP made by transformed cells is not handled as a lysosomal protein, however, since it is secreted, rather than quantitatively delivered to lysosomes as are other lysosomal proteins.

(4) Subcellular localization of MEP has been determined by light microscopic indirect immunofluorescence and electron microscopic immunolocalization using affinity-purified antibodies. In non-transformed cells, MEP localization is predominantly lysosomal, whereas in transformed cells the majority of protein is found in the Golgi region of the cells.

(5) Shortly after synthesis, the small amount of MEP in transformed cells which is not secreted is processed sequentially into two discrete lower molecular weight forms. In non-transformed cells a larger percentage of total synthesized MEP is similarly processed. At steady-state, as revealed by immunodetection of MEP on Western transfers of PAGE of unlabeled cell extracts, the lowest molecular weight antigenic form of MEP (approx. 20k) is the predominant intracellular form of the protein.

(6) We have purified mRNA coding for MEP and prepared a cDNA library from which we will isolate cloned MEP cDNAs.

Significance for Cancer Research and the Program of the Institute:  
Objective 3, Approach 3.5.

MEP is both a marker of transformation and a sensitive indicator of the presence of at least one tumor promoter. The induction of its synthesis by tumor promoters could serve as a screening test for these agents in the environment. If MEP is found to fill an essential role in tumor growth or metastasis, then specific therapy aimed at neutralizing it could be designed as a model for cancer therapy.

Proposed Course: To continue to analyze the molecular mechanism underlying induction of MEP by using cloned MEP cDNAs as probes to measure MEP mRNA levels after transformation and treatment with tumor promoters; to continue to use MEP as a marker of the molecular events involved in tumor promotion and transformation.

Publications:

Gottesman, M.M., and Yuspa, S.H.: Tumor promoters induce the synthesis of a secreted glycoprotein by mouse skin and cultured primary mouse epidermal cells. Carcinogenesis 2: 971-976, 1981.

Scher, C.D., Hendrickson, S.L., Whipple, A.P., Gottesman, M.M., Pledger, W.J. Constitutive synthesis of platelet derived growth factor modulated proteins by a tumorigenic cell line. Cold Spring Harbor Conferences on Cell Proliferation, 1982, in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08717-04 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Role of Carbohydrates in Processing and Stabilization of Glycoproteins		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Kenneth Olden, Guest Worker LMB NCI		
Others: Kenneth M. Yamada LMB NCI Head, Membrane Biochemistry Section		
Bruno Bernard, Guest Worker LMB NCI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Membrane Biochemistry Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Nonglycosylated fibronectin</u>, from tunicamycin-treated chicken embryo fibroblasts, was degraded more rapidly to acid-soluble products than <u>glycosylated fibronectin</u> by pronase, thermolysin, trypsin and chymotrypsin. The absence of carbohydrate did not markedly affect overall patterns of proteolytic fragments identified by SDS-PAGE. Except for the expected increases in electrophoretic mobilities of the nonglycosylated peptides, the only important difference was that the nonglycosylated fragment corresponding to the carbohydrate-rich, collagen-binding domain, was completely digested by the proteases in 60 min at 30°C. In contrast, the comparable fragment from glycosylated fibronectin was resistant to protease digestion. Heparin-binding domains that normally lack carbohydrate are equally susceptible to proteases in glycosylated and nonglycosylated fibronectin. Similar results were obtained by comparison of nonglycosylated <u>RNase A</u> with glycosylated <u>RNase B</u>. Also "modified" RNase B, that had undergone partial removal of carbohydrate from the protein molecule, was degraded at a rate intermediate between that observed for fully glycosylated RNase B and nonglycosylated RNase A. Finally, when glycosylation of cellular protein is inhibited, <u>cathepsin B</u>, a lysosomal protease, is secreted.         </p>		

PHS-NIH  
Individual Project Report  
October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

**Objectives:** To investigate in depth the precise role of carbohydrates in the intracellular processing, segregation and stabilization of glycoproteins.

**Methods Employed:** Fibronectin was isolated from confluent monolayer cultures by the urea-extraction procedure described by Yamada et al. (Proc. Natl. Acad. Sci. 72, 3158 and Biochem. 16, 5552). The tunicamycin-containing cultures were supplemented with 50  $\mu$ M leupeptin to increase the yield of nonglycosylated fibronectin. Glycosylated and nonglycosylated fibronectin were digested by each of four proteases (trypsin, chymotrypsin, pronase or thermolysin) in a reaction mixture containing 0.1 M NaCl, 10 mM  $\text{CaCl}_2$  and 50 mM Tris HCl (pH 7.0) at 30°C. Radiolabeled proteolytic fragments were then tested for their ability to bind to gelatin-agarose or heparin-agarose beads according to the method of Hahn and Yamada (Cell 18, 1043 and Proc. Natl. Acad. Sci. 76, 1160). Concanavalin A (Con A) Sepharose and Agarose Ricinus communis agglutinin (RCA I), equilibrated in HEPES or phosphate buffer containing bovine serum albumin, were incubated with  $^{14}\text{C}$ -labeled fibronectin and alpha-D-galactose or alpha-methylmannose. The reactive and nonreactive protein fractions were analyzed by SDS-PAGE.

Cathepsin B activity released into the culture medium was monitored by rate of hydrolysis of the radiolabeled substrate (benzoyl-L-prolyl-L-phenylalanyl-L-arginyl-analide, (analine  $^{14}\text{C}(\text{U})$ ) at pH 4.4.

**Major Findings:** To determine how the carbohydrate moiety of fibronectin influences the susceptibility of the protein to proteolytic degradation, we compared the effects of various proteases on glycosylated and nonglycosylated fibronectins. Nonglycosylated fibronectin, from tunicamycin-treated chicken embryo fibroblasts, was degraded more rapidly to acid-soluble products than glycosylated fibronectin by pronase, thermolysin, trypsin and chymotrypsin. The absence of carbohydrate did not markedly affect overall patterns of proteolytic fragments identified by SDS-PAGE. Except for the expected increases in electrophoretic mobilities of the nonglycosylated peptides, the only important difference was that the nonglycosylated fragment corresponding to the carbohydrate-rich, collagen-binding domain, was completely digested by the protease in 60 min at 30°C. In contrast, the comparable fragment from glycosylated fibronectin was resistant to protease digestion. Heparin-binding domains that normally lack carbohydrate are equally susceptible to proteases in glycosylated and nonglycosylated fibronectin. We conclude that the carbohydrate component of fibronectin plays an important role in the stabilization of a specific domain of the protein against proteolytic degradation; however, the carbohydrate does not alter overall proteolytic specificity.

Similar results were obtained by comparison of RNase A with RNase B. The native nonglycosylated RNase A protein molecule was found to be more susceptible to pronase digestion than native glycosylated RNase B. Also we found that "modified" RNase B, that had undergone partial removal of carbohydrate from the protein molecule, was degraded at a rate intermediate between that observed for fully glycosylated RNase B and nonglycosylated RNase A. The biological activities of these three protein fractions were indistinguishable.

Treatment of chick embryo fibroblasts with tunicamycin (.05 ug/ml) results in a dramatic increase (7-10 fold) in secretion of protease activity compared to control cultures. The protease activity is identified as cathepsin B-like based upon (1) substrate specificity (benzoyl-L-propyl-L-phenylalanyl-L-arginyl-<sup>14</sup>C-analine is rapidly hydrolyzed), (2) pH optimum for activity of 5.5, (3) strong inhibition by thiol reactive compounds, (4) inhibition by micro-molar concentrations of leupeptin but not by pepstatin or phenylmethylsulfonyl fluoride, and (5) by the identification of a similar activity in the lysosomal fractions isolated from homogenates of untreated CEF. Intracellular cathepsin B-like activity in TM-treated CEF is reduced to 60% of control levels after 30 h of incubation. Secretion of cathepsin B-like activity is specific and not due to leakage from damaged cells since inhibitors of protein synthesis rapidly inhibit secretion of protease activity and there is no secretion of lactate dehydrogenase after TM-treatment.

Significance for Cancer Research and the Program of the Institute: Among the most consistent biochemical changes associated with oncogenic transformation are alterations in the structural features of carbohydrate moieties of glycoproteins and the enhanced secretion of proteases. These changes may at least be partly responsible for the invasive and metastatic properties of tumor cells. However, the possible relationship of protease secretion, malignancy and specific carbohydrate structure and/or placement on glycoproteins has not been investigated. Transformed cells, with altered protein glycosylation, may be particularly interesting systems to investigate the influence of carbohydrate structural features on metabolism and secretion or proteases because of their possible involvement in malignancy. Once we know more about the role of carbohydrates, it may be possible to control the half-life of glycoproteins or to direct them to specific organs or organelles by modification of their sugar residues. Therefore, studies such as those described here may have far-reaching application in enzyme-replacement therapy in cases of enzyme-deficiency diseases, and possibly may be used to direct the transport of drugs to specific target organs or cells. Learning more about carbohydrate functions may also be useful in understanding the physiological meaning of the pronounced changes in cell-surface sugars observed during cellular differentiation and malignant transformation of normal cells.

Proposed Course: These studies will be extended by comparing the rates and mechanism of intracellular transport of nonglycosylated and glycosylated proteins in hepatic cells. We are interested in determining whether secretory glycoproteins with similar carbohydrate structures are co-transported in the same population of transport vesicles. We also plan to investigate the

molecular bases of the increased protease sensitivity of the collagen-binding domain of fibronectin.

Publications:

Maheshwari, R.K., Vijay, I.K., Olden, K. and Friedman, R.M.: Assay of glycosyltransferase activities in microsomal preparation from cells treated with interferon. Methods in Enzymol. 79: 302-306, 1981.

Olden, K., Bernard, B.A., White, S.L. and Parent, J.B.: Function of the carbohydrate moieties of glycoproteins. J. Cell. Biochem. 18: 313-335, 1982.

Olden, K., Parent, J.B. and White, S.L.: Carbohydrate moieties of glycoproteins: a re-evaluation of their function. Biochim. Biophys. Acta (Biomembrane Reviews) 650, in press.

Olden, K., Bernard, B.A., Turner, W. and White, S.L.: Effect of interferon on glycosylation of fibronectin--comparison with tunicamycin. Nature, in press.

Bernard, B.A., Yamada, K.M. and Olden, K.: Carbohydrates selectively protect a specific domain of fibronectin against proteases. J. Biol. Chem., in press.

Olden, K., Bernard, B.A., White, S.L. and Parent, J.B.: Function of the carbohydrate moieties of glycoproteins. In Glaser, L. and Fox, C.F. (Eds.): Cellular Recognition. Alan R. Liss, Inc., New York, 1982, pp. 637-659.

Yamada, K.M. and Olden, K.: Actions of tunicamycin on vertebrate cells. In Tamura, G. (Ed.): Tunicamycin. Japan Scientific Societies Press, Tokyo, 1982, pp. 119-139.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08718-04 LMB
PERIOD COVERED October 1, 1981 through September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Genetic Interaction between <u>E. Coli</u> K12 and Bacteriophage $\lambda$		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Donald L. Court, Research Biologist LMB NCI		
Others: Max Gottesman LMB NCI Head, Biochemical Genetics Section		
Gabriel Guarneros, Guest Worker LMB NCI Amos Oppenheim, Visiting Scientist LMB NCI Raghupathy Ramanathan, Guest Worker LMB NCI		
COOPERATING UNITS (if any)  Martin Rosenberg, LB, NCI		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Biochemical Genetics Section		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Bacteriophage $\lambda$ when expressed from a prophage causes several changes in host physiology ( <u>transformation</u> ). Two regions of the prophage DNA encode genes ( <u>hin</u> ) which cause this effect. One of these regions is cloned in a plasmid; one open reading frame of 288bp is contained in this fragment. The effects caused by <u>hin</u> can all be related to membrane changes in the host. The <u>regulation</u> of the $\lambda$ int gene has been determined. It can be transcribed from either of two promoters PI and PL. PI requires $\lambda$ cII protein in addition to RNA polymerase to initiate. It transcribes int and terminates at a site tI 260 bases beyond the gene. This RNA synthesizes high levels of int. PL also transcribes int but is prevented from terminating at tI by $\lambda$ N gene product which makes polymerases initiating at PL (but not PI) non-terminating. The PL transcripts do not synthesize int. A site on the PL transcript inhibits int expression. It is an endoribonuclease site ( <u>RNaseIII</u> ) located 260 bases beyond int. RNA processing here sensitizes the int m-RNA to a proposed 3' - 5' exonuclease in <u>E. coli</u> . The terminated PI transcript is not processed (the RNaseIII site is not formed) and is not sensitive to the exonuclease. This <u>posttranscriptional control</u> of int from a site located beyond the gene is called <u>retroregulation</u> .		



PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: We are interested in a type of transformation of E. coli by  $\lambda$ . To examine this problem we precisely mapped the function. Also we are studying the changes wrought on the cellular transport systems and cell membrane itself by  $\lambda$  hin expression. The hin gene is cloned on a plasmid. We will study its effect in the absence of other  $\lambda$  genes and use in vitro mutagenesis to isolate mutants of the plasmid.

Int is transcribed by the promoters  $p_I$  and  $p_L$ . Int protein is only expressed when made from  $p_I$ . Expression from  $p_L$  is blocked by a site located distal to the int gene. This site is an RNaseIII processing site and inhibition requires cells with intact RNaseIII as well as an intact site. Processing of the mRNA 250 bases beyond the structural int gene inactivates protein synthesis by increasing the degradation (presumably 3' to 5') of the int m-RNA. Host mutants in this degradation system are being sought.

The RNaseIII site in addition to being a processing site is a transcription termination site. We isolated point mutations and deletions in this region and are testing whether they affect one or both activities.

Methods Employed: Standard microbial, genetical, biochemical, and recombinant DNA techniques.

Major Findings: (1) One hin gene has been precisely mapped and the region cloned onto a plasmid.

(2) cII activates the promoters  $P_I$  and  $P_E$  to allow RNA polymerase to initiate.

(3) Int synthesis occurs from  $P_I$  transcripts that terminate at  $t_I$  260 bases beyond the int gene.

(4) Int synthesis is blocked from the  $P_L$  transcript that extends beyond the  $t_I$  terminator. Inhibition of int synthesis from this transcript is caused by RNaseIII (endoribonuclease) processing.

(5) The int mRNA of the  $P_L$  transcript is degraded when processing occurs. A 3' to 5' exonuclease has been postulated.

(6) We have defined within  $\pm 10$ bp the RNaseIII recognition site by using  $Bal31$  to generate deletion map.

(7) RNaseIII protein cuts the  $P_L$  transcript at this site in vitro. Mutants in the site are not processed.

Significance for Cancer Research and the Program of the Institute:  
National Cancer Plan Object 3, Approach 1.

In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using E. coli and  $\lambda$  as model systems. This understanding might help to prevent conversions of cells to those capable of forming cancers.

Proposed Course: (1) To determine the mechanism of host transformation by phage  $\lambda$ . 2. To determine the mechanism of transcription termination. (3) To analyse the mechanism of posttranscriptional control mechanisms, i.e., endo and exo ribonuclease control systems in E. coli.

Publications:

Miller, H.I., Abraham, J., Benedik, M., Campbell, A. Court, D.: Regulation of the integration-excision reaction by bacteriophage  $\lambda$ . Cold Spring Harbor Symposia on Quantitative Biology Volume XLV: 439-445, 1981.

Schmeissner, U., Court, D., McKenney, K. and Rosenberg, M.: Positively activated transcription of  $\lambda$  integrase gene initiates with UTP in vivo. Nature 292: 173-175, 1981.

Guarneros, G., Montanez, C., Hernandez, T., and Court, D.: Posttranscriptional control of bacteriophage  $\lambda$  int gene expression from a site distal to the gene. Proc. Natl. Acad. Sci. USA 79: 238-242, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08719-03 LMB
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PERIOD COVERED  
October 1, 1981 through September 30, 1982

TITLE OF PROJECT (80 characters or less)  
  
Development and Uses of Eukaryotic Vectors

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Bruce H. Howard	Acting Chief, GRS Section	LMB	NCI
Others:	Cornelia Gorman	Postdoctoral Fellow	LMB	NCI
	Raji Padmanabhan	Chemist	LMB	NCI
	Max Gottesman	Chief, BGS Section	LMB	NCI
	Jeff Auerbach	Guest Worker	LMB	NCI
	Sue Wickner	Chemist	LMB	NCI
	Glen Merlino	Postdoctoral Fellow	LMB	NCI
	Mark Willingham	Chief, UCS Section	LMB	NCI
	Ira Pastan	Chief, LMB	LMB	NCI

COOPERATING UNITS (if any)  
  
None

LAB/BRANCH  
Laboratory of Molecular Biology

SECTION  
Gene Regulation Section

INSTITUTE AND LOCATION  
National Cancer Institute, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 4.0	PROFESSIONAL: 3.0	OTHER: 1.0
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS      ☐ (b) HUMAN TISSUES      ☒ (c) NEITHER

☐ (a1) MINORS    ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We have used recombinant DNA techniques to construct a series of vectors which function in both Escherichia coli and mammalian cells. These constructs fall into two categories: shuttle vectors and mammalian expression vectors. Shuttle vectors are designed to permit efficient bidirectional transfer of genes between Escherichia coli and mammalian tissue culture cells. We have applied bacteriophage lambda site-specific recombination mechanisms towards developing a new class of such vectors. Mammalian expression vectors are designed to facilitate study of eukaryotic promoters, activators, and other transcriptional regulatory elements. We have constructed expression vectors which permit the the Escherichia coli enzyme chloramphenicol acetyltransferase to be used as a rapid, sensitive and specific assay for function of such elements.

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

#### PROJECT DESCRIPTION:

**Objectives:** Our objectives in this work are twofold. First, we seek to develop recombinant vectors that simplify the isolation of mammalian genes. We are particularly interested in isolating genes which control mammalian cell growth and/or the process of malignant transformation. For many such genes, currently available cloning technologies can be applied either not at all or only with great difficulty. Second, we intend to utilize the mammalian expression vectors to carry out detailed studies on the mechanisms by which genes may exert control over cell growth.

**Methods Employed:** Vectors used in recombinant DNA research must provide at least three functional elements: a selectable marker, a mode for stable propagation in the host (integration into the host chromosome or independent replication), and site(s)/capacity to accept insertion of foreign DNA sequences. The shuttle vectors which we have constructed contain two sets of these basic elements, one appropriate for E. coli and the second for mammalian cells. These may be summarized as follows: 1) selectable markers: ampicillin and/or chloramphenicol resistance for selection in E. coli; one of the currently available dominant markers for selection in mammalian cells (mycophenolic acid, aminoglycoside G-418, chloramphenicol, or methotrexate resistance). 2) mode of propagation: replication in E. coli dependent upon the bacteriophage lambda replication origin, the ColE1 replication origin of pBR322, or bacteriophage lambda site-specific integration into the E. coli host chromosome; propagation in mammalian cells dependent upon integration into high molecular weight DNA. 3) site(s) for insertion of foreign DNA sequences: unique sites occurring in bacterial resistance markers or sites created by addition of synthetic oligonucleotide linkers. Transfer of recombinant DNAs to mammalian cells is usually accomplished by the calcium phosphate precipitation method, although protoplast fusion has also been utilized.

**Major Findings:** I. Development and utilization of selectable markers. Several methods have been developed by which recombinant genomes may be introduced into and stably expressed in mammalian tissue culture cells. Calcium phosphate-DNA precipitates are avidly taken up and transiently expressed by such cells under appropriate conditions. DNA may also be introduced by polyethylene glycol-mediated fusion of cells with bacterial protoplasts or synthetic liposomes. The number of tissue culture cells, however, which continue to stably express recombinant genomes introduced by these techniques, i.e. are stably transformed, is at maximum 0.5% of the total population. In order to isolate this small fraction for further study it is thus obligatory that the introduced genome, or vector, carry a selectable marker.

The first steps in the development of vectors for the transfer of exogenous DNA into mammalian tissue culture cells involved construction of simian virus 40 recombinants that carry rabbit beta-globin cDNA coding sequences



inserted into the early region of the viral genome. From these constructs a prototypic eukaryotic vector, pSV2-beta-globin, was developed. pSV2-beta-globin combines the origin of replication and ampicillin-resistance marker from the E. coli plasmid pBR322 with a modified simian virus 40 early transcription region. This modified SV40 early region contains (in the 5' to 3' direction with respect to the direction of transcription): the SV40 early promoter, a rabbit beta-globin cDNA insert, the small t intervening sequence, and the SV40 early poly(A) addition site. The early region in pSV2-beta-globin has been further altered by deletion of 75% of T antigen coding sequences (including the T antigen AUG initiation codon) and by placement of unique HindIII and BglIII restriction endonuclease sites flanking the rabbit beta-globin insert. These unique cleavage sites render the beta-globin insert exchangeable with other coding sequences; that is, cleavage pSV2-beta-globin by HindIII and BglIII generates a linear molecule, pSV2, that carries cohesive ends for insertion of foreign coding sequences under control of the SV40 early promoter. During the period covered by this report we have either constructed or utilized the following derivatives of pSV2 that function as selectable markers by virtue of the foreign coding sequence inserted:

a) pSV2-dhfr. pSV2-dhfr carries the dihydrofolate reductase (dhfr) coding sequence from the E. coli plasmid R388. This dhfr is highly resistant to the folate antagonist aminopterin; accordingly, its transfer into and expression in mammalian cells as part of the recombinant plasmid pSV2-dhfr should confer aminopterin resistance to recipient cells. As reported previously, since growth of pSV2-dhfr transfected cells in selective medium is extremely slow, further developmental work on this system has been necessary. Vector modifications have included substitution of the promoter from the 3' long terminal repeat of Rous sarcoma virus for the SV40 early promoter, and substitution of the second intron from the human beta-globin gene for the SV40 small t intron. Variation in selective conditions have included independent titrations of aminopterin, folinic acid, hypoxanthine, and thymidine. None of these modifications has produced satisfactory growth, and experiments to improve the function of this marker are continuing.

b) pSV2-gpt. pSV2-gpt carries the xanthine-guanine phosphoribosyltransferase (gpt) coding sequence from E. coli (R. Mulligan and P. Berg, Science 209:1422, 1980). Transformation with the recombinant pSV2-gpt confers upon mammalian cells the ability to utilize xanthine as a substrate for the purine salvage pathway; thus in selective medium containing xanthine, mycophenolic acid (an inhibitor of inosine dehydrogenase), methotrexate, hypoxanthine and thymidine, this recombinant serves as a dominant selectable marker (R. Mulligan and P. Berg, Proc. Natl. Acad. Sci. USA 78: 2072-2076, 1981). The published efficiency for stable transformation with this vector is  $10^{-5}$  to  $10^{-4}$ . In the past year we have developed conditions under which CV-1 monkey kidney cells are reproducibly transformed at efficiencies of  $10^{-3}$  to  $5 \times 10^{-3}$  and NIH3T3 cells are transformed at an efficiency of approximately  $10^{-3}$ .

c) pSV2-cat. pSV2-cat carries the chloramphenicol acetyltransferase (CAT) coding sequence from the Escherichia coli transposable element Tn9. The presence of pSV2-cat in mammalian or avian cells leads to intracellular acetylation of chloramphenicol. If acetylation is sufficiently rapid, this reaction



should protect mitochondrial protein synthesis from inhibition by chloramphenicol. Recently we have shown that the CAT produced by pSV2-cat or a derivative plasmid, pRSVcat (in which the 3' LTR from Rous sarcoma virus has been substituted for the SV40 early promoter), enables at least limited growth of transfected cells under selective conditions, i.e. conditions where addition of chloramphenicol to the tissue culture medium results in uniform killing of control cells. Removal of chloramphenicol from the medium after 10-15 days permits efficient rescue of the small foci that are thought to carry CAT plasmid DNA. The wide expression range of these plasmids suggests their potential to be used as eucaryotic vectors carrying a new dominant selectable marker. Further experiments along this line are currently in progress.

d) pSV2-neo. pSV2-neo carries the aminoglycoside phosphotransferase coding sequence from the E. coli transposable element Tn5 (P. Southern and P. Berg, unpublished results). Tissue culture cells which have taken up pSV2-neo synthesize this phosphotransferase and are thereby able to inactivate the aminoglycoside G-418, an antibiotic which is toxic to most mammalian cells. We have demonstrated stable transformation frequencies with pSV2-neo of about  $10^{-3}$  for both monkey kidney CV-1 and mouse NIH 3T3 cells.

e) pSVK. pSVK carries the galactokinase coding sequence from E. coli (D. Schumperli, B. Howard, and M. Rosenberg, Proc. Natl. Acad. Sci. USA 79: 257-261, 1982). Introduction of pSVK into galactokinase-deficient R1610 Chinese hamster cells permits these cells to grow in glucose-free medium which has been supplemented with galactose. Accordingly, pSVK may be used as a selectable marker in these and, presumably, other galactokinase-deficient mammalian cells.

## II. Methods for stable transformation of mammalian cells by DNA transfection.

As described in the previous section, published efficiencies for DNA-calcium phosphate mediated stable transformation of tissue culture cells range between  $10^{-5}$  and  $10^{-4}$  (although in our laboratory efficiencies of up to  $5 \times 10^{-3}$  have been achieved). We and many other investigators are cognizant of these numbers, since they dictate the feasibility of screening recombinant libraries by transfer to and assay in mammalian cells. Over the past year the following approaches have been investigated to improve the prospects for screening libraries by this method.

a) Treatment of recipient cells with sodium butyrate. Studies in a number of laboratories have revealed that exposure of tissue culture cells to sodium butyrate (1-10 mM) causes hyperacetylation of histones and a transient, generalized derepression of gene expression. Raymond Reeves (Washington State University) first suggested that this generalized derepression might facilitate DNA-mediated stable transformation of cells either by inducing DNA recombination enzymes or by opening the structure of chromatin to facilitate recombination. Experiments in this laboratory have indicated that treatment of monkey kidney CV-1 cells with 5 mM sodium butyrate following transfection with pSV2-gpt reproducibly yields an increase in both the number and growth rate of transformants. Further experiments are in progress to determine the range of

cell types responsive to this treatment and possible contributing functions of certain plasmid structural elements.

b) Incorporation of repetitive sequences into recombinant vectors. Evidence from numerous sources suggests that DNA recombination in mammalian cells proceeds preferentially between homologous sequences. We postulated therefore that a recombinant vector carrying highly repeated sequences, because it should have a high probability of encountering homologous sequences in the host chromosome, might integrate efficiently by recombination into chromosomal DNA. To date we have incorporated the simian alpha and Alu-type repeat sequences into the vector pSV2-gpt. Experiments to evaluate the effects of these sequences on transformation efficiencies are underway.

c) Transformation of cells carried on microcarrier beads. We have found that the calcium phosphate precipitation method for introduction of exogenous DNA is strongly dependent on cell density: cultures that are in log phase growth ( $10^4/\text{cm}^2$ ) transfect with high efficiency, whereas cultures approaching confluency transfect with much lower efficiencies. The requirement that cell density be kept low makes it difficult to obtain large numbers of independent transformants without use of excessive amounts of growth medium and virtually unmanageable numbers of tissue culture plates. The advantage of microcarrier beads is that they provide a very large surface area and thus permit convenient propagation of high numbers of cells at low density. Our initial experiments indicate that efficiencies of stable transformation (using CV-1, NIH 3T3, or HeLa cells) are at least comparable to those obtained by conventional growth of cells in monolayer.

### III. Expression vectors.

pSV2-cat and derivatives. To maximize the probability that appropriate regulation of a cloned gene will be reproduced, it is often desirable to reintroduce that cloned gene into the parental cell type. Unfortunately, in the parental cell, the problem of distinguishing expression of the cloned and endogenous genes is most pronounced. One solution to this problem is to combine the transcription start site and/or putative regulatory region(s) of a cloned gene of interest with a second gene segment which provides an easily assayable and readily distinguished function. It is for this purpose that we developed the pSV2-cat system, in which just such an assayable function, the chloramphenicol transacetylase (CAT) gene, is combined with derivatives of the eukaryotic vector pSV2. There are several characteristics of the pSV2-cat system which make it uniquely useful for study of eukaryotic promoters: 1) chloramphenicol transacetylase activity is absent from most or all mammalian and avian cells. The absence interfering endogenous enzyme activities becomes particularly important in experiments where the signal from the promoter in an exogenous DNA is relatively weak and accurate quantification is desired; 2) the availability of a sensitive and convenient radioassay for this enzyme; 3) complete nucleotide sequence information on the CAT gene; and 4) the availability of rhodamine-labeled antibody to CAT. With this antibody individual cells that have taken up and are expressing recombinant CAT plasmids can be identified by immunofluorescence microscopy (Gorman, C., Merlino, G., Willingham, M., Pastan, I. and Howard, B., unpublished results).

The first promoter that we studied using the CAT system was the SV40 early promoter present in the parental pSV2 vector. Of particular interest were two 72 base pair tandemly repeated sequences in this promoter that are thought to function as activators for transcriptional initiation near the Goldberg-Hogness TATA box. We found that a mutant early promoter retaining only one of these 72 bp repeats still functions as well as the wild type promoter. On the other hand, a second mutant promoter retaining only 30% of one 72 base pair repeat sequence induces only about 11% as much CAT synthesis as the wild type promoter (about three times the background activity obtained from a plasmid that retains neither the repeat sequences nor the TATA box sequences).

We have carried out additional studies with pSV2-cat derivatives in which various eukaryotic promoters have been substituted for the SV40 early promoter region. We have found that the Herpes thymidine kinase promoter drives expression of CAT both in CV-1 monkey kidney cells and in mouse NIH3T3 cells (Gorman, Dobson, Howard, and Khoury, unpublished results). Similarly, the promoter for the chicken alpha2(I) collagen gene directs synthesis of CAT in CV-1 and chick embryo fibroblasts (Ohkubo, Gorman, Howard, and de Crombrughe, unpublished results). Less CAT is produced when these promoters replace the SV40 early promoter. In contrast, the promoter contained in the 3' long terminal repeat from Rous sarcoma virus directs 2 - 3 times as much CAT synthesis as the SV40 early promoter in both chick embryo fibroblasts and CV-1 cells. This difference between Rous sarcoma and SV40 early promoter strengths has been verified by measuring cytoplasmic mRNA after transfection of CV-1 cells (Gorman, C., Merlino, G., Howard, B., and Pastan, I., unpublished results).

Our experience from the above experiments is that the CAT system provides a rapid and accurate method to compare different promoters, to measure the apparent strength of one promoter in a number of cell types, to compare transfection protocols, and to evaluate other parameters which affect exogenous gene expression.

#### IV. The N6106/lambda-SV2 system.

We have developed the N6106/lambda-SV2 host/vector pair, a system designed to allow cloning of very large mammalian DNA fragments. Functional elements in the 8.6 Kb vector component lambda-SV2 include bacterial selectable markers for ampicillin and chloramphenicol resistance, as well as a mammalian cell selectable marker, the SV-gpt transcription unit. The ColEI replicon of the parental pSV2 vector has been replaced by the bacteriophage lambda minimum replication region; accordingly, this recombinant may be propagated in E. coli as a "lambda-dv" plasmid. A second mode for propagation of lambda-SV2 in E. coli is provided by the bacteriophage lambda attachment site; thus when this vector is introduced into the E. coli strain N6106, which contains a modified lambda cI857 prophage, it integrates directly into the E. coli chromosome by site-specific recombination. This occurs as follows: 1) N6106 is grown to mid-log phase at 32 C. (cI+, int-, xis- state), 2) the incubation temperature is raised to 42 C. for 15 minutes to induce leftward transcription from the prophage (cI-, int+, xis+ state), 3) the temperature is returned to 32 C. for 15 minutes to allow xis to decay and repression to reestablish (cI+, int+, xis- state), 4) lambda-SV2 DNA is introduced by a standard transfection proto-



col. Since replication of the vector is repressed by the prophage-encoded cI protein, stable transformation occurs only by integration into the host chromosome. The overall efficiency is 300 - 500 colonies per nanogram, and the vector may be stably maintained or "stored" in the integrated state simply by propagation at 32 C. When excision and amplification of the vector are desired, elevation of the temperature during log phase growth to 42 C. inactivates the cI repressor and permits accumulation of multiple closed circular molecules.

#### V. Shuttle vectors.

The vectors pSV2-gpt and pSV2-neo permit cloning of mammalian gene fragments in Escherichia coli, transfer of cloned fragments to mammalian cells, and selection of stable cell lines which have taken up exogenous vector DNA. In all cases that have been examined, such pSV2 recombinant genomes are found to be integrated into high molecular weight DNA (Mulligan and Berg, 1980). The apparent requirement for integration into host DNA to establish stable transformation precludes direct recovery of these recombinants for transfer back into Escherichia coli. To circumvent this problem we have modified pSV2-gpt to develop two new classes of vectors.

a) BPV/pSV2-gpt. P. Howley, Ming-Fan Law, and Nava Sarver have demonstrated that bovine papilloma virus (BPV) is carried exclusively as a multicopy episome in some mouse cell lines. In collaboration with these investigators we studied the in vivo behavior of a BPV/pSV2-gpt hybrid which contains the 69% transforming region of BPV and the SV40-Ecogpt early region transcription unit from pSV2-gpt. We found that NIH 3T3 clones selected for expression of the SV-gpt marker also undergo BPV-induced transformation, i.e. grow in soft agar, although the latter phenotype is rapidly lost in the absence of selection for gpt resistance. Dr. Howley's associates have shown that similar constructs propagate primarily as episomes, but are subject to frequent structural rearrangement. Further work with BPV/pSV2gpt-type vectors is being carried out in Dr. Howley's lab in the hope that this instability problem can be alleviated and that such constructs can be efficiently shuttled between mammalian cells and E. coli.

b) Lambda-SV2(LR). This plasmid was obtained by restriction endonuclease digestion of E. coli chromosomal DNA carrying a single, integrated copy of the vector lambda-SV2 (see section IV). Since cleavage occurred in sequences flanking the vector, lambda attL and attR sites (formed on integration of the parent lambda-SV2 into the E. coli chromosome) were retained upon recircularization of the linear restriction fragment to form the plasmid. We have introduced lambda-SV2(LR) DNA into mammalian cells under conditions which should result in formation of integrated copies flanked by attL and attR sites. Recent reconstruction experiments suggest that it will be possible to excise such integrated copies using an in vitro bacteriophage lambda site-specific recombination reaction (Auerbach, J., Wickner, S., Gottesman, M., and Howard, B., unpublished results). Excision by site-specific recombination may provide a "universal" method to rescue eukaryotic vectors for recloning in Escherichia coli.

Significance for Cancer Research and the Program of the Institute:

National Cancer Plan Objective 6, Approach 3.

1. The availability of multiple eukaryotic selectable markers and improved calcium-phosphate mediated DNA transfection techniques should extend the range of cell types which may be used for gene transfer experiments.
2. The development of new eukaryotic expression vectors based on the recombinant pSV2-cat should increase the sensitivity and accuracy, as well as reduce activators and other regulatory elements.
3. The utilization of new cloning vehicles that incorporate bacteriophage lambda site-specific recombination mechanisms should enable isolation of mammalian genes which, either by virtue of size or structural instability, cannot be carried by other recombinant vectors.
4. The development of improved eukaryotic/E. coli shuttle vector systems should permit the much more rapid cloning of mammalian genes that may be identified by their effect on growth of cells in tissue culture or by their induction of new proteins on the cell surface.

Proposed Course: Further work will concentrate on continued improvement of all aspects of eukaryotic vector function, namely, function of selectable markers, efficiency of DNA-mediated gene transfer, application to study of mammalian regulatory elements, cloning of large genes and/or complex gene loci, and shuttling between eukaryotic cells and E. coli. In addition, experiments will be initiated to apply these recombinant DNA technologies to the study of cell growth control, especially malignant transformation.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08750-02 LMB									
PERIOD COVERED October 1, 1981 through September 30, 1982											
TITLE OF PROJECT (80 characters or less)  Regulation of Regulatory Proteins in <u>E. Coli</u>											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT											
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 50%;">Sankar Adhya Head, Developmental Genetics Section</td> <td style="width: 40%; text-align: right;">LMB NCI</td> </tr> <tr><td colspan="3" style="height: 10px;"></td></tr> <tr> <td>Others:</td> <td>Susan Garges, Microbiologist Roberta Haber, Biologist Elio Gulletta, Guest Worker</td> <td style="text-align: right; vertical-align: top;">LMB NCI LMB NCI LMB NCI</td> </tr> </table>			PI:	Sankar Adhya Head, Developmental Genetics Section	LMB NCI				Others:	Susan Garges, Microbiologist Roberta Haber, Biologist Elio Gulletta, Guest Worker	LMB NCI LMB NCI LMB NCI
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COOPERATING UNITS (if any)  None											
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SECTION Developmental Genetics Section											
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205											
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SUMMARY OF WORK (200 words or less - underline keywords)											
<p>             We are studying the structure and function of several regulatory genes of <u>E. coli</u>: <u>adenylate cyclase</u> (<u>cya</u>), <u>crp</u>, <u>rho</u>, and <u>nus</u>. They regulate transcription initiation and termination of a wide variety of other operons. In order to study how these genes are themselves regulated <u>in vivo</u>, we have fused the control regions (promoter, operator, etc) of <u>cya</u>, <u>crp</u>, and <u>rho</u> genes to the gene of <math>\beta</math>-galactosidase, which can be assayed easily. Assay of the latter enzyme in these fusion strains under a variety of conditions demonstrated that all the three genes are autogenously regulated.           </p> <p>             We have also cloned the <u>cya</u>, <u>crp</u>, <u>rho</u>, and <u>nus</u> genes into phage vectors. In vitro study of the regulation of these genes using the DNA clones and purified proteins are in progress.           </p>											

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

#### PROJECT DESCRIPTION:

**Objectives:** The E. coli proteins Nus, Rho, cAMP receptor protein, and adenylate cyclase have roles in regulating the expression of many genes in the E. coli chromosome. Nus and Rho factors are involved in transcription termination and antitermination. cAMP receptor protein (CRP) acts with cAMP (the formation of which is catalyzed by adenylate cyclase) to stimulate transcription of certain operons. Our objective is to understand the nature of Nus, Rho, CRP and cAMP action.

Mutations in the genes for Rho, Nus, CRP, or adenylate cyclase result in multiple physiological defects in an E. coli cell. There is evidence that defects in the Rho protein can be counterbalanced by defects in the CRP or adenylate cyclase genes. We are suggesting that there is an inter-relationship among these regulatory proteins and plan to determine at what level (transcriptional, translational, etc) the relationship lies. In principle, we want to understand how the regulatory genes themselves are regulated and why.

**Methods Employed:** Standard microbial, genetic and biochemical techniques. Also employs both in vivo and in vitro recombinant DNA technology.

**Major Findings:** (1) The enzyme which is responsible for the synthesis of cAMP is adenylate cyclase (encoded by the cya gene). Since cAMP, along with its receptor protein, CRP, are important in the regulation of many cellular processes, then factors which modulate adenylate cyclase and CRP are necessarily important. This modulation can come about by changing the level of the proteins or by changing their activity. In order to determine whether change in cAMP/effective CRP comes about through new protein synthesis, we constructed strains which would easily enable us to measure the amounts of these proteins in the cell. Strains which produced adenylate cyclase- $\beta$ -galactosidase hybrid proteins and CRP- $\beta$ -galactosidase hybrid proteins were constructed. The production of  $\beta$ -galactosidase in these strains is under the exact type of transcriptional or translational regulation that normal adenylate cyclase and CRP would be. By measuring the readily assayable  $\beta$ -galactosidase enzyme levels in these cells, we can get an accurate reflection of the amount of adenylate cyclase and CRP. We have found that cAMP and CRP negatively regulate both crp and cya gene expression. Studies are currently planned to show if CRP and cAMP are acting as classic repressors of their own operons.

(2) We have genetic evidence suggesting that the expression of the rho gene is regulated by its own gene product (the termination factor, Rho) and by the amount of cAMP and CRP in the cell. In order to study how this regulation comes about, a strain was constructed, as in #1 above, which produces a Rho- $\beta$ -galactosidase hybrid protein. Using this method to determine

indirectly the amount of Rho in the cell, we have found that rho is autoregulated at the level of transcription or translation. Furthermore, cAMP and CRP act to negatively regulate the amount rho in the cell. Currently, in vitro studies are being performed to determine the level at which these regulations come about.

(3) We have continued characterization of the previously cloned E. coli fragment which contains the rho gene. This cloned fragment, when present, complements rho mutants so that they appear as wild type. There are two proteins encoded by this fragment. One is Rho protein but the other's identity and location on the fragment is unknown. We are investigating clones which delete certain portions of the fragment to determine if the small unknown protein has any significance in complementing mutant rho phenotypes.

(4) We have cloned a fragment containing the gene (cya) which encodes adenylate cyclase from E. coli. We are currently characterizing this fragment and will use it to study, in vitro, how CRP/cAMP are regulating cya expression (see #1).

(5) We have incorporated a previously isolated clone of the crp gene into a phage vector in order to study its in vivo regulation.

(6) We have also cloned genes coding for the NusA protein, which modulate transcription termination. We plan to study its own regulation.

Significance for Cancer Research and the Program of the Institute:  
National Cancer Plan Objective 3, Approach 1. In cancer cells the expression of some genes are permanently turned on, i.e., expressed constitutively and some genes are permanently turned off, i.e., never expressed. Our studies are aimed to understand the molecular basis of how genes are turned on and off and how genetic regulatory elements interact with each other. This understanding might help to prevent conversion of normal cells to those capable of forming cancers.

Proposed Course: (1) To determine the complete structure of the regulatory genes of E. coli: rho, cya, crp, nus. (2) To determine the regulation of the expression of these genes. (3) To understand the interaction between these genes.

#### Publications:

Adhya, S., Garges, S., and Ward, D.F.: Regulatory circuits of bacteriophage  $\lambda$ . In Cohn, W. (Ed.): Progress in Nucleic Acid Res. Acad. Press, 1981, Vol. 26, pp. 103-118.

Macchia, V., Caputo, G., Mandato, E., Rocino, A., Adhya, S., and Pastan, I.: Guanylate cyclase activity in E. coli mutants defective in adenylate cyclase. J. Bact. 147: 931-934, 1981.

Adhya, S., and Garges, S.: How cAMP and its receptor protein act in E. coli. Cell, 1982, in press.

Gulletta, E., and Adhya, S.L.: Cloning of the rho gene of E coli. Microbiologica, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08751-02 LMB															
PERIOD COVERED October 1, 1981 through September 30, 1982																	
TITLE OF PROJECT (80 characters or less)  Genetic Regulatory Mechanisms in <u>Escherichia Coli</u> and its Bacteriophage																	
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SUMMARY OF WORK (200 words or less - underline keywords)  <p>             We are studying the modulation of the transcription processes in <u>E. coli</u>, using the <u>gal</u> operon and phage lambda as model systems. We have defined so far the functional behavior of several regulatory elements--cyclic AMP, CRP, Rho, Nus and N--that modulate transcription. We have evidence which suggest that each of these elements modulate gene transcription in more than one of the following ways: Activation of initiation, Inhibition of Initiation, Activation of termination, Inhibition of termination. The molecular mechanisms are currently being investigated.           </p> <p>             In our continuing effort to characterize the complete regulatory features of the <u>gal</u> operon, we have now identified the in vivo transcription initiation sites of the <u>gal</u> mRNA from the two (p1 and p2) promoters of <u>gal</u> both by sizing the mRNAs and by capping the triphosphate ends with labeled 32P-GTP and then sequencing the labeled RNaseT1 fragments. We have also discovered a locus within the first structural gene that regulates <u>gal</u> transcription.           </p>																	



PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objectives: We believe that the expression of most genes in E. coli is regulated in one way or another. Qualitatively different mechanisms have been discovered, e.g., positive and negative control at the level of both initiation and termination of gene transcription. Control mechanisms also exist at the level of mRNA processing and translation. We have demonstrated many of these control mechanisms using the galactose (gal) operon of E. coli and its phage lambda. Using these model systems, we are studying the molecular basis of the following regulatory mechanisms: (1) How cyclic AMP and CRP catalyze/inhibit transcription initiation. (2) How Rho and Nus proteins of E. coli modulate transcription termination. (3) How lambda N gene product catalyzes transcription antitermination. (4) How gal repressor inhibits transcription initiation. (5) The nature of protein-protein and protein-nucleic acid interactions that bring about the above control mechanisms.

Methods Employed: Standard microbial, genetic and biochemical techniques. Also employs both in in vivo and in in vitro recombinant DNA technology.

Major Findings: (1) The gal operon in E. coli consists of three structural genes, K, T, and E, the operator-promoter region being located at the E end. The operon has two promoters, p<sub>1</sub> and p<sub>2</sub>. The expression of the various gal cistrons are modulated by an interplay of the two gal promoters, an operator, gal repressor, RNA polymerase and cyclic AMP and its receptor protein. We have identified the in vivo start sites of gal mRNA from p<sub>1</sub> and p<sub>2</sub> by capping the triphosphate ends with labeled 32P-GTP and then sequencing the labeled RNase T1 fragments. These sites are identical to the corresponding points demonstrated in vitro.

(2) We have previously developed a novel in vivo binding assay for isolating gal operator mutants. It involves in vitro mutagenesis of a small fragment of the gal repressor molecules. Plasmid mutants (o<sup>c</sup>) unable to bind the gal repressor have been isolated as inducible cells. These mutations have been studied in vivo by their introduction into chromosomal DNA by genetic manipulations, developed in our laboratory. The mutations have also been studied by DNA sequencing. One class of mutations map in the cyclic AMP-CRP binding region indicating an interaction between cyclic AMP-CRP complex and gal repressor on gal DNA. The other class maps within galE the first structural gene, suggesting a novel control in the gal operon. We are currently studying the o<sup>c</sup> mutations in vitro.

(3) We have been investigating the mechanism of action of lambda antitermination function, the N gene product. Our approach involves studying host mutants which affect N gene activity. In collaboration with David Friedman of the University of Michigan, we have identified the products of

some of the host mutants (nus), which prevent N antitermination activity. nusA gene codes for factor L and nusE gene codes for ribosomal protein S10. This was shown by first cloning the nusA and nusE genes and then identifying the corresponding products made both in vivo and in vitro. These findings are consistent with our previously proposed hypothesis that translational apparatus modulate transcription termination reactions. We are currently studying the site of interactions of nus gene products in their role to achieve transcription antitermination.

(4) We are currently investigating the question whether the nus, rho, cya, and crp gene products are essential for the cell. For this purpose, we are isolating conditional lethal or chain-terminating mutations in the above genes. Our results already indicate the essential nature of rho and nus genes.

Significance for Cancer Research and the Program of the Institute:  
National Cancer Plan Objective 3, Approach 1. In cancer cells, the expression of some genes are permanently turned on, i.e., expressed constitutively. Our studies are aimed to understand the molecular basis of how genes are turned on and off. We are using gal and  $\lambda$  as model systems. This understanding might help to prevent the conversion of normal cells to those capable of forming cancers.

Proposed Course: (1) To determine the complete structure of the gal operon. (2) To identify the regulatory molecules involved in turning on and off the various genes of the gal operon. (3) To understand the biochemistry of transcription initiation and termination signals. (4) To elucidate the mechanism of action of the N-gene. (5) To understand the protein-protein interactions that modulate the transcription complex.

#### Publications:

Friedman, D.I., Schauer, A.T., Baumann, M.R., Baron, L.S., and Adhya, S.L.: Evidence that ribosomal protein S10 participates in control of transcription termination. Proc. Natl. Acad. Sci. USA 78: 1115-1118, 1981.

Merrill, C., Gottesman, M.E., and Adhya, S.L.: E. coli gal operon proteins made after prophage lambda induction. J. Bact. 147: 875-887, 1981.

Adhya, S., and Irani, M.: Regulatory aspects of the galactose operon of E. coli. In Genetic Approaches to the Study of Metabolic Processes. The University of Iowa, 1981, pp. 8-9.

Adhya, S., and Gottesman, M.E.: Promoter Occlusion: Transcription through a promoter may inhibit its activity. Cell, 1982, in press.

Busby, S., Irani, M. and deCrombrughe, B.: Isolation of mutant promoters in the E. coli galactose operon using local mutagenesis on cloned DNA fragments. J. Mol. Biol. 154: 197-209, 1982.

Aiba, H., Adhya, S., and deCrombrughe, B.: Evidence for two functional gal promoters in intact E. coli cells. J. Biol. Chem. 256: 11905-11910, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER Z01 CB 08752-02 LMB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Mechanism of the Transport of Thyroid Hormones into Animal Cells		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	Sheue-yann Cheng	Research Chemist      LMB      NCI
Others:	Ryuya Horiuchi Ira Pastan Mark Willingham	Visiting Associate      LMB      NCI Chief, LMB      LMB      NCI Chief, UC Section      LMB      NCI
COOPERATING UNITS (if any)  None.		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION National Cancer Institute, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.1	PROFESSIONAL: 2.1	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Using 3,[125-I]3',5-triiodo-L-thyronine ([ <sup>125</sup> I]T3), the binding and uptake of thyroid hormone in cultured Swiss 3T3-4 and GH3 cells were characterized. The results showed saturable, reversible and stereospecific binding of T3 to both cell types. The results also showed that the uptake of T3 is <u>receptor mediated</u> . Using <u>affinity labeling techniques</u> , a protein with a molecular weight of 55,000 was specifically labeled in both cell types. The 55,000-dalton protein is postulated to be involved in mediating the uptake of T3 into cells. Using <u>electron spin resonance</u> , the dynamic interactions of <u>thyroid hormones</u> with <u>liposomes</u> derived from L- $\alpha$ -dimyristoyl-phosphatidylcholine was studied. The <u>rotational</u> and <u>lateral diffusing</u> of L-thyroxine in liposomes was determined.		

PHS-NIH

Individual Project Report

October 1, 1981 through September 30, 1982

PROJECT DESCRIPTION:

Objective: To elucidate the mechanism of the entry of thyroid hormones into cells at molecular level. To study the mechanism of the translocation of thyroid hormone from cytoplasm into nuclei where the initiation of biological actions occurs. To understand the mechanism of thyroid hormone action on cell growth.

Methods Employed: Synthesize rhodamine-labeled 3,3',5-triiodo-L-thyronine ( $T_3$ ) and L-thyroxine and study their binding and internalization in cultured fibroblasts and GH3 cells by video-intensification fluorescence microscopy. Covalently conjugate  $T_3$  to peroxidase, IgG, biotin or dinitrophenyl derivatives and study their intracellular localization by electron microscopy. Use radio-labeled  $T_3$  to quantify the binding and internalization of thyroid hormone by cells. Synthesize photoactivable and chemical affinity labeling reagents to covalently label the plasma membrane receptor which will be purified and characterized. Thyroid hormones labeled with nitroxide radicals are synthesized. Electron spin resonance spectroscopy is employed to study the dynamic interactions of thyroid hormones with plasma membrane  $T_3$  receptors of intact cells.

Major Findings: It was shown earlier that the entry of  $T_3$  into cultured fibroblasts occurs by receptor-mediated endocytosis. Using radiolabeled  $T_3$ , the entry of  $T_3$  into cells and the subsequent incorporation of the hormone into nuclei was shown not only to be inhibited by monodansylcadaverine but also by bacitracin, substances that block the receptor-mediated endocytosis of  $\alpha_2$ -macroglobulin. The results showed not only that receptor-mediated endocytotic entry of  $T_3$  to cells is physiologically significant, but also that this mode of entry is probably operating, in addition to cultured mouse fibroblasts and rat GH3 cells, in other cell types.

Affinity labeling of the purified plasma membrane of GH3 cells with N-bromoacetyl-[ $^{125}$ I] $T_3$  (BrAc[ $^{125}$ I] $T_3$ ) identified a  $T_3$ -specific membrane receptor with a molecular weight of 55,000. Proteins with the same molecular weight were also specifically labeled by BrAc[ $^{125}$ I] $T_3$  in intact GH3 cells and Swiss 3T3 fibroblasts. These findings suggest that the 55,000-dalton protein appears to be involved in mediating the entry of  $T_3$  into cells.

Nitroxide-labeled L-thyroxine and  $T_3$  were synthesized. Electron spin resonance spectroscopy was used to probe the motional characteristics of thyroid hormones in liposomes. The effective rotational correlation time of spin-labeled thyroxine in dimyristoyl-phosphatidylcholine multilamellar vesicles were determined as  $3.1 \times 10^{-9}$  sec (the linear term) and  $4.8 \times 10^{-9}$  sec (the quadratic term). The lateral diffusing coefficient was estimated to be  $5.2 \times 10^{-8}$  cm<sup>2</sup>/sec. These parameters will be compared with those determined in intact cells. The information derived from this technique will provide new insights into the nature of the hormone-receptor dynamic interactions at molecular level.



Significance for Cancer Research and the Program of the Institute: National Cancer Plan Objective 5, Approach 5.

Transformation of cultured cells appears closely linked with the ability to form malignant tumors in vivo. The understanding of the basic mechanisms in hormone-receptor interactions which results in controlled or uncontrolled cell-growth are likely to be of great value in understanding the basic mechanisms leading to the formation of cancer cells. The knowledge gained from such studies is likely to have great impact on the ability to design or modify therapeutic procedures.

Proposed Course: We will continue to study the molecular mechanism of the transport of thyroid hormones into animal cells. We will use the current methodologies or develop new biochemical techniques to explore the mechanisms of thyroid hormones in affecting cell growth, thereby gaining new insights into the understanding of the precise mechanism by which cancer cells are able to grow uncontrollably.

Publications:

Edelhoch, H., Cheng, S.-y., and Itrace, N.: Spectroscopic properties of rhodamine B-labeled thyroid hormone. Annal. N.Y. Acad. Sci. 366: 253-264, 1981.

Pastan, I., Haigler, H., Dickson, R., Cheng, S.-y., and Willingham, M.: The role of the receptosome in receptor-mediated endocytosis. In Mozes, L., Schultz, J., Scott, W.A., and Werner, R. (Eds.): Cellular Responses to Molecular Modulators. Miami Winter Symposia Series. New York, Academic Press, 1981, Vol. 18, pp. 137-148.

Horiuchi, R., Cheng, S.-y., Willingham, M., and Pastan, I.: Inhibition of the nuclear entry of 3,3',5-triiodo-L-thyroxine by monodansylcadaverine in GH<sub>3</sub> cells. J. Biol. Chem. 6: 3139-3144, 1982.

LABORATORY OF PATHOPHYSIOLOGY  
ANNUAL REPORT SUMMARY  
October 1, 1981 to September 30, 1982

The research effort of the Laboratory of Pathophysiology is focused on the following subjects:

1. Pathophysiology of mammary gland and tumors.
2. Biochemical changes related to onset and cessation of lymphocyte proliferation.
3. Structure and function of biological membranes.

Three more projects with a limited involvement in terms of manpower are carried on: (a) Cachexia in tumor-bearing animals, (b) Effects of ionizing radiations on DNA, proteins and bone marrow, and (c) Modification of amino acid molecules as an approach to find antitumor agents.

1. Pathophysiology of mammary gland and tumors

(1a.) Gene organization of secretory proteins in normal and neoplastic mammary epithelium: (P.K. Qasba, P. Chomczynski, S. Safaya and E. Appella)

Four projects are being carried on: (a) Sequence analysis of cDNA clones for  $\alpha$ -LA, whey and K-protein. Rat  $\alpha$ -LA was found to be bigger than any known  $\alpha$ -LA. It has 17 extra residues beyond the COOH terminus, the sequence is hydrophobic, proline rich and bears some resemblance to  $\beta$  casein. Rat whey proteins are rich in cysteine, glutamic acid, aspartic acids and serine but lack tyrosine. The cysteines are repeated in two domains and the second domain has striking similarities with the second domain of the red sea turtle protease inhibitor. The encoded K-protein lacks cysteine and has several potential phosphorylation sites. The mammary gland of the pregnant animal shows a steady increase in demethylation of  $\alpha$ -LA, Wp and K-gene sequences until during lactation when these genes are totally demethylated. In tumors, the pattern of demethylation varies and does not resemble any stage of mammary gland secretion. (b) Structural gene organization of  $\alpha$ -LA and the DNA sequence analysis of a genomic clone is being studied. Rat  $\alpha$ -LA clones have been isolated from a rat liver DNA library. Arrangement of coding and intervening sequences have been established. The gene is about 4 Kb long, about 5 times the length of the structural gene. (c) MTW9 rat mammary tumors transcribe the genes coding for  $\alpha$ -LA, K-protein 42K and 25K caseins. The whey phosphoprotein and X-casein are not or very poorly transcribed. Isolated nuclei synthesized in vitro the milk protein specific mRNAs. After hormonal deprivation of the host, the nuclei of regressing MTW9 reduce incorporation of UTP and RNAs showed lower levels of polyadenylation. (d) In rat mammary gland explants insulin and hydrocortisone are indispensable for production of  $\alpha$ -LA and whey phosphoprotein mRNAs. Prolactin enhances the effects of insulin and hydrocortisone, but it is not indispensable for the mRNAs production. Hydrocortisone seems indispensable for prolactin action on mRNA production. K-protein mRNA was not produced unless prolactin was present with insulin and hydrocortisone.

(1b.) Cyclic nucleotides in growth regulation of mammary tumors: (Y.S. Cho-Chung, T. Hasuma, C. Kapoor, F. Huang, T. Clair, B. Berghoffer, C. Shephard, and J. Katz)

The study of hormone-dependent mammary tumor regression has progressed along 3 major directions and the following observations were made: (a) During regression obtained with a variety of treatments (ovariectomy, DBcAMP, cholera toxin or anti-

estrogens) the cAMP receptor level was consistently enhanced while the receptor level for estrogen was consistently decreased in an antagonistic manner. Within 6 hr post-ovariectomy or DBcAMP treatment, the in vitro synthesis of several polypeptides instructed by poly(A)<sup>+</sup>RNA of the tumor was consistently changed regardless of the regression effector. One protein band MW 20.5K was expanded and two others (MW 22K, 35K) were reduced. The changes did not occur when hormone independent tumors were treated in a similar way. The response observed in vivo could be reproduced in vitro on tumor slices. (b) Carcinogenic doses of 7-12 DMBA failed to induce mammary carcinomas in rats receiving DBcAMP. A marked decrease in the binding of [<sup>3</sup>H]DMBA to mammary gland DNA was observed in vivo after oral administration of DBcAMP. Incubation of mammary gland slices with <sup>3</sup>H DMBA in the presence of DBcAMP showed a reduction by 50% of the carcinogen to DNA and the presence of DMBA inhibited DNA-binding of <sup>3</sup>H DBcAMP. Prevention of DMBA-induced mammary carcinogenesis by DBcAMP may be due to blocking of the carcinogen binding to DNA. (c) Antibodies against bovine type I and II cAMP-dependent protein kinase subunits (R<sup>I</sup>, R<sup>II</sup>) were raised in rabbits and were purified by utilizing affinity chromatography. The antibodies specifically cross-reacted with human neoplastic mammary cells. Immunocytochemical procedures utilizing these antibodies showed that R<sup>II</sup>, but not R<sup>I</sup>, is located in the nucleoli and mitotic spindles. Photoaffinity labelling of the proteins of MCF7 mammary cells revealed the presence of 50K isoprotein of type II cAMP-dependent protein kinase.

(lc.) Changes occurring in cell populations during differentiation: (W.B. Anderson, R. Gopalkrishna, A. Kraft, L. Nagarajan, and C. Jaworski)

OTT6050, a stem cell line derived from a mouse teratoma and two sublines F9 and PYS were utilized in these experiments. The following findings have been obtained during the past year: (a) A new rapid method for purification of calmodulin was developed utilizing hydrophobic interaction chromatography. (b) Retinoic acid induced differentiation of F9 cells into endoderm-type cells and produced a time-dependent increase of a cytosolic protein kinase dependent on calcium and phospholipids for its activity. (c) Retinoic acid treatment of F9 cells enhanced both cytosolic and plasma membrane-associated cyclic AMP-dependent protein kinase activity. (d) As differentiation into endodermal cells progresses, adenyl cyclase of F9 cells become unresponsive to calcitonin but increased cyclic AMP production under parathyroid-hormone stimulation. (e) Undifferentiated F9 cells secrete immunoreactive calcitonin while differentiated endoderm cells secrete immunoreactive parathyroid hormone. (f) A 55K protein present in several viral and carcinogen transformed cells was also formed in OTT6050 and the F9 subline. As differentiation into endodermal cells progresses the 55K protein disappears. (g) Insulin promoted growth of F9 cells. This action appears to occur via insulin receptors present in F9 cells not through MSA receptors also present in F9 cells. The effort of the group is now oriented toward a coordination of these and previous observations into a biologically meaningful picture.

(ld.) Growth and differentiation of normal and neoplastic mammary cells: (W. R. Kidwell, J. Zwiebel, M. Bano, M. Purnell, S. Taylor, K. Burdette, J. Shaffer, W. Lewko, and D. Salomon)

Three areas have been emphasized: (a) Studies on dietary lipids and breast cancer revealed that prolactin stimulates the release of free fatty acids from the mammary adipocytes. Histamine appears to be an intercellular signal which functionally couples adipocytes to the epithelium of the gland. Histamine triggers the adipocyte to release fatty acids. Mast cells, very abundant in the mammary gland, are probably the source of histamine. Removal of mast-cells from a preparation of mammary epithelium blocked the histamine release by prolactin. (b) Production of type IV collagen has been previously found to be essential for



monitoring growth of mammary epithelium, normal and neoplastic. The work of the past year resulted in the isolation of the cells synthesizing collagen in the mammary adenocarcinomas. These tumors produce 2 factors which stimulate collagen synthesis. The factors have MW 68K and 6K and appear to act on collagen mRNA rather than affecting collagen elimination. A variety of hormones and growth factors able to stimulate growth of mammary epithelia were found also to differentially increase collagen synthesis; except cholera toxin. (c) The importance of poly ADP-ribose synthetase in DNA repair has been further studied. It was found that the synthetase activity was blocked by heat shock with a loss of cell viability by 5-25%. Repair of DNA singlestrand breaks takes place in the absence of poly ADP-ribose synthetase. 3-acetyl aminobenzamide has been synthesized and found to be a very powerful inhibitor of poly ADP-ribose synthetase. The compound also blocked growth of CHO cells and caused death of mouse embryos in utero. The role of poly ADP-ribose synthetase in cell growth is now being examined.

(1e.) Effects of prolactin on mammary and ovarian cells: (R.A. Knazek, A. Rotondi, J. Karanian, S. Liu, and J.R. Dave)

Three areas have been emphasized: (a) Starting from observations reported in previous years that PRL receptors are inducible even when protein synthesis is blocked, the relationship between membrane fluidity and detection of PRL receptors was studied. PRL-binding increased 60% in hepatocytes incubated with phospholipase A<sub>2</sub> or lysolecithin but decreased about 60% when the mice were in a diet depleted of essential fatty acids. Block of prostaglandin synthesis by indomethacin also reduced existing PRL-binding sites in a dose-responsive fashion. In hypophysectomized animals physiologic replacement doses of PRL caused an increase in PGF<sub>2</sub> $\alpha$  and PGE synthesis as well as membrane fluidity. Experimental data in support of a correlation between PRL binding and membrane fluidity were established not only for the liver but also for primary mammary tumors induced by DMBA and NMR. Hormone manipulations that reduced PRL in serum increased membrane rigidity, reduced PRL-receptors and enhanced PGE and PGF<sub>2</sub> $\alpha$  synthesis. An assay to measure the level of prostaglandin receptors has been developed. An increase of binding capacity but with unchanged K<sub>d</sub> was observed in DMBA and NMU induced mammary tumors after ovariectomy. Copper ions were also found to enhance the number of PGE binding sites of cell membranes. (b) From observations listed in previous reports the hypothesis was formulated that modification of PG metabolism could influence ovulation. Data have been accumulated during the past year to support that the euprolactinemic and the hyperprolactinemic states modify ovarian steroidogenesis and the ovulatory process by either accentuating or suppressing the FSH-related stimulation of prostaglandin synthesis. (c) The capillary culture technique, developed in this laboratory, was applied to study intracellular pH and energy metabolism using NMR and HTC<sub>10</sub> and A6 cells. The artificial capillaries in the perfusion unit have been modified to permit the analysis. Data are not yet available.

(1f.) Structure and biological function of molecules constituting the basement membrane. Effects of metastasizing cells thereon: (L. Liotta, T. Kalibec, N. Rao, and R. Russo)

Human  $\alpha$ -thrombin specifically hydrolyzed the  $\beta$  subunit of laminin. The  $\alpha$  subunit of laminin was purified and obtained in the native form. EM studies of laminin and the isolated subunit enabled identification of the long arm (78 nm) with the 400 KD chain and the  $\alpha$ -subunit with 3 similar 200 KD chains. The terminal globular domains on the small arms of the  $\alpha$ -subunits are required for tumor cells to attach to type IV collagen. Several fractions digest the  $\alpha$ -subunit of laminin

and all produce a single fragment that was purified. This fragment specifically binds to the tumor cell surface which has a receptor-like domain for laminin binding. A specific glycoprotein has been identified that mediates the attachment of tumor cells to the basement membrane.

Dr. Liotta's group was the first to find that type IV and type V collagens are degraded by specific collagenases. Type IV collagenase has been purified and its cleavage products have been partially characterized. Secretion of this collagenase is directly related to the metastasizing capacity of neoplastic cells. An antiserum against type IV collagenase is being prepared. A neutral metal protease has been identified which cleaves native type V collagen under conditions where pepsinized type IV collagen or interstitial collagens are not degraded. The enzyme is secreted in culture by malignant macrophages. The partially purified enzyme exists in a latent form requiring trypsin activation, has a molecular weight about 80 KD, is inhibited by EDTA but not by phenyl methylsulfonyl fluoride and produces specific cleavage products of both A and B collagen chains.

A test was developed to measure the ability of cells to penetrate across the basement membrane of human amnion. Migration of leucocytes, M5076 and Ewing's sarcoma across the amnion has been evaluated. The unexpected observation that interferon enhances the penetration of Ewing's sarcoma cells across the amnion has been made. The basement membrane of human amnion appears also to be an excellent substrate to grow adult rat hepatocytes.

(lg.) Growth and differentiation of mammary gland: (B.K. Vonderhaar, T. Horn, M. Bhattacharjee, and A. Greco)

The research activity was oriented toward 3 major projects: (a) The nature of lactogenic hormone receptors and factors which affect hormone binding to this molecule have been studied using Con A and S-adenosyl-L-methionine to modify protein aggregation and fluidity of the membrane. Lactogenic hormone binding to membrane is increased when fluidity is enhanced and decreased when fluidity is reduced. Con A recognizes a protein that binds lactogenic hormones. Since Con A does not affect receptors for EGF or somatogenic hormones, it is hypothesized that changes in membrane fluidity involved localized regions.

Purification of the prolactin receptor from livers of lactating mice has been achieved and preparation of an antiserum is well underway. The receptor was found to be 57-61 KD; to represent a complex of PRL with a single peptide of MW  $37000 \pm 2000$ ; to retain its specificity after purification and to bind  $^{125}\text{I}$ -oPRL with a  $K_d$  of  $2-6 \times 10^{-9}\text{M}$  as does the particulate form. (b) The roles of prolactin, thyroid hormones, adrenal steroids and EGF were analyzed in whole gland cultures. Mouse  $\alpha$ -lactalbumin was isolated from lactating glands. An estimated MW of 14600 was obtained for the purified preparation which is constituted by one protein with 2 peaks of enzyme activity. Mouse  $\alpha$ -LA appears to exist in 2 charged forms, both equally active in the lactose synthetase assay. Antisera to mouse  $\alpha$ -LA was produced in rabbits and an RIA was developed able to detect 0.25 ng  $\alpha$ -LA. The influence of T<sub>3</sub> was analyzed in whole organ cultured in media with insulin, hydrocortisone and prolactin. T<sub>3</sub> had no influence on casein production;  $\alpha$ -LA produced by the gland formed 2 peaks collected separately in 12% SDS-PAGE; only one was secreted. Tunicamycin failed to reveal that differences in glycosylation were responsible for the presence of the peaks.

The optimal concentration of hydrocortisone in the media of whole gland culture was found to be  $10^{-6}\text{M}$  for casein synthesis but for  $\alpha$ -LA was between



$10^{-8}$  and  $10^{-7}$ . The disparity was eliminated when the natural adrenal corticosterone and aldosterone were used in place of hydrocortisone. Aldosterone is necessary for lobule alveolar development of mammary glands in culture when a preliminary priming period of 9 days with  $E_2$ /Prog is done in vivo. The priming time can be shortened if EGF is added to the cultures, however, an EGF increment is not detected in the gland primed in vivo. It is hypothesized that a mammary growth factor which competes for EGF receptors is being produced during in vivo priming, necessary for labulo-alveolar development. (c) C3H/HeN mice were raised on a fatty acid deficient diet in the attempt to obtain adult animals with an atrophic mammary gland. The objective was to influence the development of the atrophic gland in adult mice. Treatment with a variety of hormones would permit to relate frequency of mammary oncogenesis with hormonal milieu present during gland development. After an initial success, it has been impossible to obtain consistently mice sufficiently healthy and with atrophic glands.

(lh.) Angiogenesis and tumor growth: (P.M. Gullino, K. Raju, F. Grantham, M. Ziche, and G. Allesandri)

In previous years it has been observed that the capacity to induce new formation of vessels is acquired by mammary and other cell populations during neoplastic transformation, but before the unrestrained growth was an acquired and stable characteristic of the population. The mechanism of the angiogenic event is being studied with the double purpose of developing an assay that can detect acquisition of angiogenic capacity in biopsies and therefore have possibilities of predicting high risk of neoplasia and finding a way of interfering with tumor growth by interfering with the neoformation of vessels which is indispensable for tissue growth. The data obtained thus far sustain the following conclusion: (1)  $PGE_1$  is a strong angiogenesis effector; (2) Copper ions concentrate in the tissue prior to invasion by capillaries; (3) Molecules of different species can become effectors of angiogenesis; (4) Ceruloplasmin is a natural angiogenic molecule provided that it carries copper; (5) None of the known effectors of angiogenesis can mobilize capillary endothelium in vitro except the complex Heparin + copper. Presently, our working hypothesis on the mechanism of angiogenesis is the following:  $PGE_1$  -- ceruloplasmin fragments carrying copper -- binding of  $Cu^{II}$  to heparin -- mobilization of capillary endothelium. Work aimed at characterizing each step and verifying their relevance in vivo is underway.

(li.) Growth factors and tumor promoters in cell growth and differentiation: (D.S. Salomon, M. Paneerselvam, and K. Smith)

(a) Several experimental approaches are being followed to study the modulation of cellular growth and differentiation by EGF and a mammary tumor growth factor. EGF can modulate the synthesis and turnover of type IV collagen in a human epidermoid carcinoma cell line (A431) and a mouse embryonal carcinoma line (EC). Phorbol esters can inhibit binding of  $^{125}I$  EGF to cell surface receptors on EC cells. The high affinity receptor for phorbol ester is, however, distinct from the EGF receptor. The role of these receptors in modulating growth and extracellular matrix formations by A431 and EC cells is being investigated. (b) The serum-free media of 7-12 DMBA primary cultures contains a mammary tumor growth factor (MTF) able to compete for  $^{125}I$  EGF binding to EC cells. MTF is antigenically distinct from EGF, induces growth of NRK in soft agar, and is mitogenic for NRK, RME, CEF and 3T3 cell lines. MTF level is highest in hormone dependent mammary tumors and lowest in transplanted tumors. It is present in human mammary gland obtained from mammaplasty. Epithelial and myoepithelial cells produce MTF. MTF is constituted by 2 polypeptides 68 K pI  $\approx$  5.2 and 6 K pI  $\approx$  7.6. Purification, characterization and antibodies preparation are underway.

## 2. Biochemical changes related to onset and cessation of lymphocyte proliferation.

(2a.) Regulation of protein synthesis during lymphocyte proliferation and response of lymphocytes to biologically active substances: (H.L. Cooper, R. Braverman, N. Feurstein, and D. Monos)

Four areas have been emphasized during this year: (a) Eight specific proteins (I-peptides) are induced by purified interferon in human lymphocytes. Different normal donors require different doses of interferon for maximal induction of I-peptides. This variable sensitivity may be related to clinical responsiveness. This possibility is now being tested on patients with mammary carcinomas. (b) About 4 hr after growth stimulation of lymphocytes, activation of a site-specific enzyme system occurs which recognizes a single lysine-containing region of a cytosolic protein  $M_r \sim 17$  KD and  $pI \sim 5.1$ . A modified lysine is produced [ $N^{\epsilon}$  (4-amino-2 hydroxybutyl) lysine] called hypusine. This modification of lysine occurs in growing cells of several types examined. The protein is presently purified and used to produce monoclonal antibodies.

(c) Resting lymphocytes are prominently synthesizing the HLA-ABC heavy chain ( $M_2$ -43 KD) and its accompanying light chain 82 microglobulin. These protein, restricted to the cell membrane, do not increase during phytoemagglutinin stimulation as much as other proteins do. By quantitating these changes in purified lymphocytes subpopulations, two objectives are being pursued: to establish whether mainly T or B lymphocytes are involved in the production of these proteins and to assess whether the protein pattern is specific for each individual. (d) A human promyelocytic cell line (HL-60) is induced to differentiate into monocytes by phorbol 12-myristate-3-acetate. As part of the differentiation process cell motility and protein production increased. The protein synthesis was restricted to the membrane fraction and specifically involved the monocytes. Characterization of these surface proteins is ongoing.

(2b.) From gene to protein: structure function and control in eukaryotic cells: (S.L. Berger, W. Eschenfeldt, R. Puskas, K. Daruwalla, D. Wallace, and L. Liotta)

A cDNA sequence coding for human immune interferon has been identified in a cDNA library prepared from gel-fractionated messenger RNA obtained from human, mitogen stimulated lymphocytes. The DNA sequence codes for a polypeptide of 166 amino acids, 20 of which could constitute a signal sequence. The polypeptide produced by expressing this DNA sequence in *E. coli* or cultured monkey cells has properties characteristic of  $\gamma$ -interferon. The effect of interferons on the invasiveness *in vitro* of Ewing's sarcoma was evaluated. When treated with either crude or homogenous fibroblast or lymphoblastoid interferon for 6 days, the cells responded by producing type IV collagenase at levels 2 to 10-fold higher than control values. The ability of Ewing's sarcoma cells to invade human amnion connective tissue was also determined. After 6 days of exposure to crude leukocyte, homogenous lymphoblastoid or homogenous fibroblast interferon, invasiveness of the treated samples increased 3-, 17-, and 22-fold, respectively, relative to that of untreated controls.

## 3. Structure and function of biological membranes.

(3a.) Tight junction structure dynamic topology: (P. Pinto da Silva, S. Barbosa, A. Aguas, M. Torrissi, and C. Parkison, J. Chevalier, and G. Tadvaikar)

Two major projects are carried on: (1) Analysis of our and previous experimental data lead to proposal that tight junction strands consist of inverted cylindrical micelles sequestered in an apolar domain. We also propose for the

useful in radiation therapy. The past year work includes electron spin resonance studies of spin trapped free radicals produced by  $\gamma$ -radiolysis in the polycrystalline state and in aqueous solutions of amino acids, peptides and nucleic acid constituents. Radicals were also generated by ultra violet photolysis dibenzol peroxide photoinduced reactions and ultra sound. Conclusive evidence for the formation of hydroxyl-radicals and of hydrogen atoms in cavitation bubbles during sonolysis of aqueous solutions has been obtained by spin-trapping and electron spin resonance.

Ms. Delta Uphoff continued her work on physical factors that may affect the radiobiological effects on inbred strains of mice, in particular, on the success of bone marrow transplantation. Physical factors heretofore considered of little consequence have been found to be critical for the reproducibility of results. New parameters for "defining" x-ray treatment are being sought to better characterize experimental conditions and results.

Modification of amino acid molecules as an approach to find antitumor agents:  
(T. Otani and M. Briley)

N-benzoyl derivatives, especially those of phenylalanine were the most effective growth inhibitors of the host-bacillus casei 7469, selected as test system. 77 halo-benzoyl halophenylalanine derivatives were found active and 14 of them had an  $ID_{50}$  of 0.33 M or less which is 2 to 7 times more powerful than 6 mercaptopurine. The most powerful growth inhibitor thus far prepared has been p-iodo-benzoyl-p-iodo-DL-phenylalanine. The inhibitory effect seems due to a suppressive action on enzymatic activities such as carboxypeptidase and acylase.



first time that the occluding functions of this junction are conferred by the fusion and continuity of the exoplasmic halves of the plasma membranes from both cells. The tight junction is proposed to represent a stable structural intermediate of membrane fusion. (2) Rapid, massive proliferation of tight junction elements can be induced by very short (2 minute) periods of osmotic shock of toad bladders in vitro. The dynamics of the process and of its reversal were investigated as well as the effect of cytoplasmic perturbors.

(3b.) Freeze-fracture cytochemistry: labelling of plasma and intracellular membranes.

The unusual promise new "fracture-label" techniques developed in our laboratory is shown by application to the localization of lectin receptors in a variety of plasma and intracellular membranes: (1) human erythrocyte membranes: we show that, upon fracture glycophorin, a transmembrane glycol protein partitions preferentially with the exoplasmic half whereas Band 3 (also a transmembrane protein) partition preferentially with the protoplasmic half. We propose a stochastic mechanism for the fracture behavior of transmembrane proteins. (2) Fracture-labeling of Con A receptors in Acanthecuseba cells shows for the first time the localization and distribution of glycolipids in plasma and intracellular membranes. We show that these lipids are confined to exoplasmic membrane halves. (3) Topology of sialoglycoconjugates and other glycoproteins is a highly polarized cell (pig and human sperm) that demonstrates the existence of domains of transmembrane sialoglycoproteins, an existence heretofore unsuspected in and revealed here by "fracture-label." (4) Comparative topology of glycoproteins and glycolipids in natural and virally transformed cells. Initial fracture label results indicate reductions in the expression of transmembrane proteins in virally transformed cells. (5) Identification of subpopulations of T lymphocytes by fracture label. Human T cells plasma membranes are shown to have different amounts of transmembrane sialoglycoproteins as revealed by labelling of WGA receptors by fracture-labelling techniques. (6) Topochemistry of membrane glycoproteins and glycolipids in ovary-dependent mammary carcinomas reveals increase of transmembrane glycoproteins in the plasma membranes of cells from regressing tumours. (7) Fracture-labelling of human platelets reveals the unsuspected existence of numerous WGA receptors in the plasma membranes of Bernard Saulier platelets and shows, for the first time, the distribution of these lectin binding sites in human normal, thromastenic and Bernard Saulier platelets.

Cancer cachexia: (S. Morrison and E. McDuffie)

The feeding response to reduced environmental temperature is depressed by the presence of a tumor but the depression is independent of tumor binders. The reactive hypophagia and loss of body weight that normally occurs on withdrawal of insulin is attenuated in the presence of a tumor. This may be a practicable procedure for combating cachetic anorexia. The efficacy of enteral and parenteral feeding is being investigated as well as the relationship between satiety and sedation and discrimination between attenuated hunger and premature satiety as an approach to understand hypophagia induced by cancer. Final results of collaborative work with Dr. Ahrens on imposed exercise and carbohydrate source on tumor growth are being evaluated.

Effects of gamma irradiation on nucleic acids and proteins: chemotherapy agents and radiation: (P. Riesz, K. Makino, M. Mossoba, and A. Carmichael)

The modification of radiation damage of DNA by cancer chemotherapy agents of the intercalating and alkylating types is studied to collect information

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00306-19 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Modification of Amino Acids in Search of Possible Anti-tumor Agents		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Theodore T. Otani                      Research Chemist                      LPP, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Nucleic Acids Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.0	PROFESSIONAL: 1.0	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES                      X <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) a) To prepare <u>new amino acids and amino acid analogs</u> and their derivatives by <u>organic chemical methods</u> in search of compounds that <u>destroy cancer cells</u> . b) To isolate, characterize and chemically modify possible antitumor agents from biological sources.		



## Project Description:

Objectives: To prepare chemical compounds which preferentially destroy cancer cells by mechanism other than those of agents presently being used in cancer chemotherapy.

The project, initially concerned with developing simple methods of the chemical preparation of  $\beta$ -hydroxyamino acids and with the study of the biochemical properties of these compounds, has been directed in recent years to the search of chemotherapeutic agents active against cancer. The interest has been centered primarily on amino acids, amino acid analogs, and their derivatives thus far, but will be extended to other classes of compounds.

In the past 3 years the studies have centered on substituted benzoyl derivatives of ring-substituted phenylalanine analogs.

The immediate objectives of the project have been: (a) to prepare various substituted N-acyl derivatives of amino acids and amino acid analogs in various combinations of the N-acyl group and the amino acid moiety, and (b) to study the relationship between inhibitory activity and the structure of these compounds, as the combinations of the acyl groups and the amino acid analog moieties are varied, and (c) to test these compounds for anti-tumor activity, first in a microbial anti-tumor pre-screen system and, if found to be active, in a mammalian tumor system.

Methods Employed: In most cases, acylation, including aroylation, was accomplished by the Schotten-Baumann procedure, which involves addition of the acylating agent in a 2-phase system sufficiently basic to expose the amino group. In some cases, this was modified by dissolving the appropriate acylating agent in an organic solvent. The contaminating bi-product, the substituted benzoic acid, was generally removed from the reaction mixture by fractional crystallization from carbon tetrachloride, and the product was further purified by crystallization from ethanol, or by precipitation from ethanol-water. In some cases, crystallization was accomplished by repeated cycles of treatment with ethanol, acetone, and petroleum ether.

Microbiological assay was carried out in a riboflavin-supplemented riboflavin assay medium, and the extent of inhibition was determined turbidimetrically. The test organism was Lactobacillus casei 7469.

Major Findings: Among many acyl amino acid analogs we have studied, the N-benzoyl derivatives, especially those of phenylalanine and phenylalanine analogs were the most potent inhibitors of the microbial system selected. When a systematic study including 72 compounds consisting of 12 substituted benzoyl derivatives of 5 ring-substituted phenylalanine analogs was undertaken, it became apparent that certain groups in certain positions in the benzoyl ring and in the phenyl ring of phenylalanine favored increased inhibitory activity. These results suggested the possibility that halo-substitution in the phenyl ring of both the benzoyl moiety and of the phenylalanine moiety in the benzoylated phenylalanines would result in compounds of greater inhibitory capacity.

1. With this in mind, the ortho, meta, and para substituted fluoro-, chloro-, bromo-, and iodo-benzoyl derivatives of ortho-, meta-, and para-substituted

fluoro-, chloro-, bromo-, and iodo-phenylalanine were prepared. This included the synthesis of 77 compounds. The purity of each of these compounds was ascertained by (a) elemental analysis, (b) melting point determination, and (c) gasometric determination of free amino nitrogen.

2. All 77 of these halo-benzoyl halo-phenylalanine showed considerable inhibitory activity, the extent of inhibition meeting the criteria of positivity of the protocol of microbial anti-tumor screen described by Foley, et al.

3. When compared on an equimolar basis, all but 6 compounds showed complete or nearly complete inhibition at 4.47 mM. The 6 compounds not showing 100 percent inhibition also exhibited considerable inhibition, i.e., from 54-78%.

4. Determination of the  $ID_{50}$  values of the most inhibitory compounds in the L. casei system showed that 14 compounds had  $ID_{50}$  values of 0.33 mM or less, which is some 2 to 7 times more powerful than 6-mercaptopurine, a compound that is presently used in cancer therapy.

The 14 compounds are: m-chlorobenzoyl derivatives of p-bromo- and p-iodo-DL-phenylalanine, p-chloro derivatives of p-bromo- and p-iodo-DL-phenylalanine, m-bromobenzoyl derivatives of p-chloro, p-bromo-, and p-iodo-DL-phenylalanine, p-bromobenzoyl derivatives of p-bromo- and p-iodo-DL-phenylalanine, o-iodo-benzoyl derivatives of p-iodo-DL-phenylalanine and the p-iodo-derivatives of o-chloro-, p-chloro-, p-bromo- and p-iodo-DL-phenylalanine.

5. In general, the m-chlorobenzoyl-, p-chlorobenzoyl-, m-bromobenzoyl-, p-bromobenzoyl-, and p-iodobenzoyl- derivative of m-chloro-, p-chloro-, m-bromo-, p-bromo- and p-iodo-phenylalanine are the most potent inhibitors in the system used. The most powerful inhibitor thus far prepared in these studies is p-iodobenzoyl-p-iodo-DL-phenylalanine.

6. Utilizing the information gained from these studies, attempts were made to synthesize the p-bromobenzoyl- and p-iodobenzoyl derivatives of amino acid analogs known to be effective in cancer therapy (melphalan) and against cancer cells (mimosine). Thus far, only the crude form of the first has been made. Purification of the compounds was met with considerable difficulty so far. The preparation of the halobenzoylated derivatives of mimosine has not been successful thus far. The difficulty could be due to the fact that mimosine does not behave as typical amino acid chemically and physically.

7. The microbial anti-tumor prescreen system using L. casei 7469 has been up-dated. Fifteen anticancer agents presently used in cancer therapy, or in the developmental phase in cancer therapy, were tested in this system. In preliminary studies, the screening system was capable of detecting 13 of the 15 compounds tested. The 2 compounds not detected was probably missed by this system because the form in which they were tested may not have been the active form but may possibly require metabolic modification to an active form by the host.

8. Preliminary studies investigating the mechanism of inhibition by this group of inhibitors have shown that they are capable of inhibiting certain isolated enzyme systems, i.e. carboxypeptidase and acylase.

Significance to Biomedical Research and the Program of the Institute: That amino acid metabolism may be a susceptible area of action in cancer therapy was demonstrated by the efficacy of asparaginase in certain tumors which required asparagine. Amino acid deprivation, either by the removal of the amino acid or by the substitution with an analog may be effective in inhibiting tumor growth.

The enhancement of activity of amino acid, and amino acid analogs upon acylation appears to be of importance in the cancer program in view of the fact that one serious drawback in cancer chemotherapy has been that of the inaccessibility of the cytotoxic agent to the cell. It is possible that alteration of the molecular charge by acylation, and the introduction of a cytotoxic group, in the form of certain acyl radicals, has provided a molecule that is more permeable to the cell and at the same time, once within the cell, is capable of releasing the cytotoxic agents by the action of intracellular acylases. Objective 6, Approach 2.

Proposed Course of Research:

1. The preparation of p-halobenzoyl derivatives of amino acid analogs now being used in cancer therapy will be continued. In addition to the p-iodo-benzoyl derivatives of melphalan, p-halobenzoyl derivatives of other amino acid analogs active against cancer cells, such as mimosine, azaserine, 6-diazo-5-oxo-norleucine, and alanosine will be synthesized.

2. The study of the mechanism of inhibition by this class of compounds using isolated enzyme system will be continued. In addition to carboxypeptidase, and acylase presently being studied, aminotransferase will be studied.

3. The study of the up-dating of the microbial antitumor prescreen will be continued. There is much interest in this study at the present time because of a probable curtailment of funds for the screening program and the need for rapid and inexpensive means of screening for probable anti-cancer compounds.

4. A search for anti-cancer agents in molds will be initiated.

5. The preparation and antitumor studies of antimetabolic amino acid analog coupled with essential fatty acids will be continued.

Publications:

Otani, T.T. and Briley, M.R.: Structure-activity relationships among substituted N-benzoyl derivatives of phenylalanine and its analogs in a microbial antitumor prescreen. I: Derivatives of o-fluoro-DL-phenylalanine. J. Pharm. Sci. 71: 214-216, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 00941-26 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Genetic and Other Factors Affecting Marrow Transplantation in Irradiated Mice		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Delta Uphoff                      Research Biologist                      LPP, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology SECTION Radiation Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.0	OTHER: 2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The investigation of <u>physical factors</u> affecting biological experiments was continued. These experiments included both the <u>radiobiological effects</u> on different inbred strains of mice and the effect of various physical factors on the success of bone marrow transplantation experiments. Physical factors, heretofore, considered of little consequence when dealing with biological material have been demonstrated to be critical for the reproducibility of these experiments in other laboratories. New parameters for "defining" x-ray treatment are required along with more precise reporting of experimental conditions.		



## Project Description:

Objectives: To investigate genetic and physical factors influencing the success of bone marrow replacement following the administration of toxic levels of ionizing irradiation and/or chemotherapeutic agents used in cancer treatment. The ultimate goal is to devise methods for preventing or controlling lethal graft-versus-host reactions (GVHR) while utilizing possible immunotherapeutic benefits of less severe reactions. To accomplish this goal it has been necessary to investigate and reevaluate many of the universally accepted concepts and practices used by radiobiologists.

Methods Employed: Twenty-six inbred strains and substrains of mice representing the major histocompatibility genotype (H-2) and especially derived substrains are bred and maintained in a closed colony for use in this investigation. Since the mice used for experimentation are residual animals not required for the maintenance of the breeding colony, they are continually available in relatively small numbers and constitute more males than females. For the most efficient utilization of the mouse supply and animal room space, different types of experiments are run concurrently. Following exposure to lethal total-body irradiation from either the double tube 250 kVp therapeutic x-ray machine or the two directional cesium-137 irradiator each mouse receives an intravenous inoculation of bone marrow cells of different genetic disparities between the H-2 genes and immune response genes as well as sex and parity status. In addition, various treatments are given to marrow donors or to the cell suspension to determine the effect of treatment on the protective capacity of the marrow inoculation and on the GVHR.

Major Findings: Among the physical factors already demonstrated to effect the results in this biological system were the quality of the X-rays emitted by two different X-ray machines, using the same filtration and operated under conditions that result in the same half value layer (HVL) of 0.9 mm. Cu. The HVL is universally used to "describe" the quality of X-rays used. It has been assumed that X-rays from machines producing X-ray of the same HVL would produce the same biological effects. However, this assumption has now been demonstrated to be incorrect. At all doses below the lethal range for which comparative data are available using both machines, the Westinghouse Quadrocondex X-ray machine used between 1948-1976 produced both greater and earlier mortality than the presently Philips RT250 machine presently in use under the same experimental condition using mice from the same closed colony. Following a lethal exposure, mortality curves for irradiated controls coincide. However, survival of marrow grafted mice exposed to the same lethal dose from the 2 X-ray machines differed significantly depending upon the genetic relationship between donor and recipient. Among the genetic combinations available there were different survival patterns representing either changes in the protective capacity of the marrow as indicated by a shift in mortality during the first two weeks post irradiation, or a shift in the onset and/or severity of the GVHR. The dose differential has not yet been thoroughly investigated. However, this experimental system is sufficiently sensitive to detect an increase of as little as 25R Preliminary data for one combination of strains indicates that an increase greater than 25R would be required to duplicate the survival pattern obtained with the Westinghouse X-ray machine.



Alternate exposure similar to those used in clinical therapy to decrease the surface exposure while increasing the midline dose were tested. Since the best therapeutic results seem to be obtained when the radiation through multiple portals is administered on the same day, the alternate exposures to mice were administered with minimum time lapse between the two exposures. Thus, the two alternate exposures and the single dorsal and ventral exposures were administered during essentially the same protracted time. Even without the added variable of a time lag between exposures the results differed. The alternate dorsal + ventral exposures resulted in greater survival than the single dorsal exposure and conversely, when ventral exposure preceded dorsal exposure more animals died than with ventral exposure alone but fewer animals died than with the dorsal + ventral exposures. A critical requirement for demonstrating this phenomenon is the separation of data for males and females. This paradox is not easily explained. The uniformity of mid-line tissue dose may account for the difference between two directional simultaneous exposure and the unidirectional exposure but does not explain the observed difference between the two alternate exposures. The exposure times are so short that repair during exposure should not be a significant factor. Marrow transplantation following alternate exposure has not yet been attempted. However, extrapolating from the available data on simultaneous vs. dorsal exposures followed by marrow inoculation, the survival of marrow inoculated mice exposed to dorsal + ventral irradiation would be expected to differ from those exposed to ventral + dorsal irradiation.

In addition to a difference in survival of high leukemic AKR mice exposed to alternate irradiation there is preliminary data suggesting that the incidence of leukemia following ventral + dorsal x-irradiation is greater than following dorsal + ventral irradiation. It will be of interest to determine whether the type and direction of the exposure will affect in incidence and genotype of the leukemias arising in AKR mice inoculated with low leukemic CBA marrow.

To test the validity of the effect of direction of the exposure observed following x-irradiation similar experiments are being conducted using the Gamma-cell-40 two directional cesium-137 irradiator that was modified to allow for the use of alternate and single sources. Mouse holders are placed on a trivet so that the midline of the mouse is at the midline of the chamber. With the gamma irradiation the differences between two directional, dorsal and the dorsal + ventral exposures do not appear to be as great as those following x-irradiation, i.e., the time factor may be less evident with the higher energy irradiation although the dose rate of 129R/minute is comparable with that of the x-irradiation (126 R/min.). In contrast the difference between dorsal and ventral exposures appear to be greater than that observed following x-irradiation. There is sufficient data available using the cesium source to indicate that directional effects of exposure observed with x-ray are not an artifact.

The high leukemic AKR strain has been used for donors and recipients for reciprocal experiments involving low leukemic CBA mice. After treatment with 1050 R two-directional gamma irradiation only two lymphomas developed in the AKR mice. The first developed 5 months after treatment and proved to be of AKR origin by the transplantation test. The second developed 8 months

after treatment and was a CBA neoplasm. No neoplasms have developed in the CBA recipients of AKR marrow. The higher energy gamma irradiation from Cs-137 has a greater advantage over x-rays in that less gut damage occurs even at much higher doses so there is a minimum of early mortality occurring before the transplanted marrow has become functionally established. Even so a larger group of mice will be required before the comparative incidence of leukemia can be determined. These experiments have temporarily been curtailed in order to complete experiments using the X-ray machine.

#### Significance to Biomedical Research and the Program of the Institute:

Supposition does not become fact by repetition. Progress in science depends upon the replacement of old concepts by new facts. The reluctance to replace accepted dogma as a result of experimental results that cannot be explained in conventional terms has been one of the biggest deterrents to progress in radiation biology. The first requirement for establishing new facts is the reproducibility of experiments in other laboratories. Failure to reproduce experimental data results in pat answers of all variations ranging from "dirty mice" to "inaccurate calibration" depend upon what best fits the particular situation. Now more fundamental reasons for the failure to reproduce experimental data must be considered. For example, the HVL, which has been used to "describe" the x-ray beam for purposes of standardizing the output of x-ray machines, is no longer adequate. More precise description of the radiation equipment and the experimental conditions will be required than has been customary heretofore. However, few laboratories, including our own, are equipped to test for changes in the wave length distribution that produce significant changes in the results of animal experiments without changing the HVL or calibrated dose as is observed with the addition of an auxiliary voltage regulator to the X-ray equipment. Other factors affecting the quality of the absorbed dose that have the potential to effect the results are scatter from thick plastic animal holders or conditions of maximum backscatter, used to decrease the exposure time when one tube is used. These experiments have potential importance for all projects using ionizing irradiation since they directly effect the reproducibility of the experiments.

The direction of the exposure is a critical factor for both x-irradiation and gamma irradiation from Cs-137. The availability of dual opposing x-ray equipment is probably rare. This is one possible explanation for why some of our marrow transplantation experiment have not been readily reproduced elsewhere; i.e., the two directional Westinghouse unit produced a higher and earlier mortality thus mice for marrow transplantation were exposed to a lower total body dose than that customarily used in other laboratories. In the case of cesium irradiators the direction of the exposure becomes of paramount importance. Cesium irradiators, unlike x-ray machines, should produce the most consistent results between different laboratories. However, there are at least two quite different designs for these irradiators. Although the quality of irradiation is not the variable in this case, these irradiators should not be expected to produce comparable results. The Gammacell-40, two directional unit is produced by AECL and should give the most uniform tissue dose particularly if the mouse holder is placed on a trivet the

midline of the mouse corresponds to the midline of the irradiation chamber. In contrast, the MarkI unit by Shephard has a single source located at the side and back of the irradiation chamber. With this lateral exposure variables would be less readily monitored and controlled and a mean tissue dose will be administered.

Proposed Course of Research: The comparative effect of two-directional, dorsal, ventral and alternate exposures and the dose dependence of this phenomenon will be completed using the Gammacell-40 cesium source. Bone marrow transplantation in high leukemic AKR mice will be expanded included the type and direction of the exposure to X-rays as they effect the incidence and genotype of the leukemias arising in high leukemic recipients of marrow from low leukemic strains. Efforts will also be made to reproduce and expand experiments involving involving maternal influences using gamma irradiation for comparison with data previously obtained using the 250 kV x-ray machines. Whether the basic radiobiological experiments can be successfully phased out in favor of the immunogenetic investigations of the type conducted before the Westinghouse x-ray machine was dismantled in 1976 will depend upon the response obtained when our data are submitted for publication.

Publications:

Uphoff, D.E. In Memoriam Egon Lorenz: 30 years ago. In Baum, S.J. and Lechrey, G. D. (Eds.): Experimental Hematology Today 1981. Springer-Verlag, New York, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 00942-22 LPP																
PERIOD COVERED <b>October 1, 1981 to September 30, 1982</b>																		
TITLE OF PROJECT (80 characters or less) <b>Effects of gamma-irradiation on nucleic acids and proteins</b>																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																		
<table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 30%;">P. Riesz</td> <td style="width: 30%;">Chief, Radiation Biol.,</td> <td style="width: 10%;">LPP, NCI</td> </tr> <tr> <td>Others:</td> <td>K. Makino</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td></td> <td>M. M. Mossoba</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td></td> <td>A. Carmichael</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> </table>			PI:	P. Riesz	Chief, Radiation Biol.,	LPP, NCI	Others:	K. Makino	Visiting Fellow	LPP, NCI		M. M. Mossoba	Visiting Fellow	LPP, NCI		A. Carmichael	Visiting Fellow	LPP, NCI
PI:	P. Riesz	Chief, Radiation Biol.,	LPP, NCI															
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TOTAL MANYEARS: <b>3.5</b>	PROFESSIONAL: <b>3.5</b>	OTHER:																
CHECK APPROPRIATE BOX(ES)																		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER																		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																		
SUMMARY OF WORK (200 words or less - underline keywords) <p>           The effects of <u>ionizing and ultra-violet radiation</u> on nucleic acids and proteins and their constituents are being studied. The modification of radiation damage in DNA by cancer chemotherapy agents of the intercalating and alkylating types is of interest, since such information may be useful in <u>radiation therapy</u>.         </p> <p>           The present report includes <u>electron spin resonance (e.s.r.) studies of spin-trapped free radicals</u> produced by <u>γ-radiolysis</u> in the polycrystalline state and in aqueous solutions of <u>amino acids, peptides and of nucleic acid constituents</u>. Radicals were also generated by <u>ultra violet photolysis, dibenzoyl peroxide photoinduced reactions and ultrasound</u>. Conclusive evidence for the formation of hydroxyl radicals and of hydrogen atoms in cavitation bubbles during sonolysis of aqueous solutions has been obtained by spin trapping and e.s.r.         </p>																		



Project Description:

**Objectives:** The effects of ionizing and ultraviolet radiation on biological macromolecules and their constituents are being investigated. For reproductive death, DNA is the target molecule in viruses and is at least a part of the target molecule in bacteria and mammalian cells. Radiation damage to DNA is produced by the "direct effect" through the formation of radical ions, electrons, excited states and neutral free radicals or by the "indirect effect" where radical species formed in the surrounding medium by radiation react with DNA. For water, these species are hydrated electrons, hydrogen atoms and hydroxyl radicals. In the case of radiation damage to the chromosome, radicals formed in the nucleohistone may cause damage to the DNA.

In the chain of events that leads to loss of biological activity, free radicals play an important role. Chemical compounds have been discovered which significantly modify radiation effects. These include: (a) electron affinity sensitizers which act on hypoxic tumor cells, (b) halogenated pyrimidines which are incorporated into DNA and (c) cancer chemotherapy agents of the intercalating or alkylating type which sensitize tumor and normal cells.

Studies of the mechanism of action of radio-sensitizers and radio-protectors are necessary to design improved combinations of chemotherapy and radiation therapy.

An understanding of the mechanisms by which ionizing radiation brings about the loss of biological activity in macromolecules is likely to help in the development of new methods for altering the efficiency of cell killing with possible benefits to radiation therapy.

In the last few years it has become apparent that superoxide anion radicals and hydroxyl radicals are found in many biological systems in the absence of either ionizing radiation or UV-photolysis. Recent reports have indicated that hydroxyl radicals are produced in the presence of certain anti-cancer drugs such as bleomycin and adriamycin. The significance of radical reactions is therefore not confined to radiation biology.

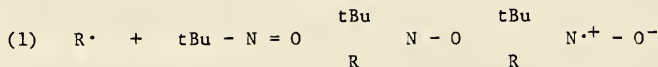
**Methods Employed:** Nucleic acids, proteins and their constituents were  $\gamma$ -irradiated either in the solid state at  $2 \times 10^{-5}$  torr or in aqueous solutions in an 800-curie Cobalt  $\gamma$ -source. Electron spin resonance studies were carried out with a Varian E-9 Spectrometer connected to a Nicolet Lab 80 computer. For photolysis studies at specific wavelengths, a 1000-watt high pressure Mercury-Xenon arc source and monochromator were employed.

**Spin-trapping Method:**

In the spin-trapping method, the short-lived free radicals react with a diamagnetic scavenger (the spin-trap) to produce longer-lived radicals (the spin-



(the spin-adduct) which can be conveniently investigated by e.s.r. In our studies, t-nitrosobutane (tNB) was employed as the spin-trap, equation (1):



The e.s.r. spectrum of the spin-adduct nitroxide shows a primary triplet splitting due to the  $^{14}N$  nucleus and secondary splittings which usually arise from the magnetic nuclei of the trapped radical  $R^{\bullet}$ . From the e.s.r. spectra of the nitroxide, the structure of  $R$  can often be identified. For some experiments in which several radicals were spin-trapped simultaneously, the resulting spin-adduct nitroxides were separated by means of a Waters High Pressure Liquid Chromatograph with a C<sub>18</sub>-micro BONDAPAK column and water-methanol elution gradients. A capillary flow-detector in the e.s.r. cavity was used to indicate the positions of the free radical peaks.

Major Findings: 1. Photoinduced reactions of dibenzoyl peroxide as studied by e.s.r. and spin-trapping (with I. Rosenthal and M.M. Mossoba). The photochemical reactions of dibenzoyl peroxide with some organic compounds were found by e.s.r. and spin-trapping to generate free radicals in dimethyl sulfoxide solutions at room temperature. Two reaction mechanisms occur which determine the structures of the radicals generated. The first involves a one-electron oxidation and the second a hydrogen atom transfer. The prevailing mechanism is primarily dependent on the structure of the substrate. With carboxylic acids the one-electron oxidation occurs exclusively, leading to the loss of the loss of the carboxyl group and to formation of the alkyl radical. For alcohols both alkoxy radicals and hydrogen-abstraction  $\alpha$ -carbon radicals were spin trapped. The alkoxy radicals were generated by the electron transfer mechanism. Finally, pyrimidine bases such as thymine and cytosine yielded C(5)-centered radicals which could also be explained by an electron transfer mechanism. These observations are of interest because of the recently observed skin tumor-promoting activity of dibenzoyl peroxide. 2. Spin-trapping with 2-methyl-2-nitrosopropane: Photochemistry of carbonyl-containing compounds. Methyl radical formation from dimethyl sulfoxide (with I. Rosenthal and M. M. Mossoba). The photochemical reactions of several carbonyl-containing compounds investigated by spin-trapping with 2-methyl-2-nitrosopropane revealed different modes of scission depending on the structure of the initial compound. Thus, in photo-Fries rearrangements, the acyl radical was detected. 1,3-diphenyl-2-propanone decarbonylated to yield the benzyl radical. Finally, valerophenone yielded the radicals expected by  $\gamma$ -hydrogen abstraction. In a dark reaction, dimethyl sulfoxide reacts with NaOH to generate methyl radicals. The latter result suggests the need for caution in the use of dimethyl sulfoxide with 2-methyl-2-nitrosopropane for the detection of hydroxyl radicals. 3. Electron spin resonance of spin-trapped radicals of amines and polyamines. Hydroxyl radical reactions in aqueous solutions and  $\gamma$ -radiolysis in the solid state (with M.M. Mossoba and I. Rosenthal). The reactions of hydroxyl radicals with methylamine, dimethylamine, trimethylamine, diethylamine, sec-butylamine, ethylenediamine, 1,3-diaminopropane, putrescine, cadaverine, 1,7-diaminoheptane, ornithine, spermidine, spermine, agmatine, and arcaine in aqueous solutions have been investigated by spin-trapping and e.s.r. Hydroxyl radicals were generated by the uv photolysis of  $H_2O_2$  and

2-methyl-2-nitrosopropane (MNP) was used as the spin-trap. The effects of ionizing radiation on the same polyamines in the polycrystalline state were also investigated. The free radicals produced by  $\gamma$ -radiolysis of these solids at room temperature in the absence of air were identified by dissolution in aqueous solutions of MNP. The predominant reaction of OH with amines and polyamines below pH 7 was the abstraction of hydrogen atoms from a carbon that is not adjacent to the protonated amino group. For agmatine and arcaïne which contain guanidinium groups abstraction occurred from the  $\alpha$ -CH.

Dimethylamine was oxidized to the dimethylnitroxyl radical by  $H_2O_2$  in the dark.  $\gamma$ -Radiolysis of polyamines in the polycrystalline state generated radicals due to H-abstraction from either the  $\alpha$ -CH or from a carbon atom in the middle of the alkyl chain. The deamination radical was obtained from ornithine. 4. Photochemistry of protein and nucleic acid constituents: electron spin resonance and spin-trapping with 2-methyl-2-nitrosopropane (with I. Rosenthal). Our recent studies of the direct uv-photolysis of aliphatic and aromatic peptides, DNA constituents, and their 5-haloderivatives in aqueous solution and the photo-induced reactions of benzoylperoxide with amino acids, peptides, fatty acids, and pyrimidines in dimethylsulfoxide-containing solutions were summarized. 2-Methyl-2-nitrosopropane was used for spin-trapping and characterization of free radicals generated photochemically with light in the wavelength range of 220-313 nm in aqueous or aprotic solvents. Direct photolysis of aliphatic dipeptides and of phenylalanine peptides produced mostly decarboxylation radicals, while for tyrosine peptides both decarboxylation and deamination radicals were spin-trapped: for tryptophan di- and tripeptides, deamination radicals were predominantly produced, while for long chain polypeptides, main-chain scission was observed. When pyrimidine bases were photolysed, radicals consistent with the addition of an H-atom or an OH-radical at the C(5) position of the 5,6-double bond could be detected. The general reaction pattern in the photolyses of 5-chloro, bromo, or iodouracil was the homolytic cleavage of the carbon-halogen bond, while for 5-fluoracil, the  $\alpha$ -fluoro radical was spin-trapped. Dibenzoylperoxide was found to photoinduce the free radical generation in amino acids, peptides, and fatty acids exposed to ultraviolet light, which is not absorbed by these compounds, that is  $\lambda = 313 \pm 10$  nm. The most predominant reaction is the decarboxylation of the terminal acid moiety. This process is explained by an electron transfer from the acid to the photo-excited peroxide or its fragmentation products. Pyrimidine bases, such as cytosine and thymine, can be oxidized under these conditions to generate C(5) centered radicals. 5. Electron spin resonance of spin trapped radicals in  $\gamma$ -irradiated polycrystalline dipeptides. Chromatographic separation of radicals. (with K. Makino). Polycrystalline dipeptides (glycyl-glycine, glycyl-L-valine, glycyl-L-leucine, L-alanyl-glycine, and L-prolyl-L-alanine) were  $\gamma$ -irradiated at room temperature in the absence of air. Subsequently they were dissolved in aqueous solutions containing 2-methyl-2-nitrosopropane as the spin trap. From the e.s.r. spectra of the nitroxide radicals separated by high-performance liquid chromatography, structural assignments of the radicals were made. For glycyl peptides, H-abstraction for the  $\alpha$ -carbon atoms of the carboxyl terminal residues and from the side-chains were observed. For L-alanyl-glycine, H-abstraction from the glycyl residue and the formation of the deamination radical could be shown to occur. For L-prolyl-L-alanine, the ring opening (deamination) reaction, decarboxylation and H-abstraction from the C-terminal  $\alpha$ -carbon were seen.

6. E.s.r. of spin-trapped radicals in  $\gamma$ -irradiated polycrystalline amino acids. Chromatographic separation of radicals (with K. Makino). The free radicals produced by  $\gamma$ -radiolysis of polycrystalline amino acids (L-valine, L-leucine,

L-isoleucine and L-proline) at room temperature in the absence of air were investigated by spin trapping with 2-methyl-2-nitrosopropane (MNP). The spin adducts produced by dissolving the irradiated solids in aqueous MNP solutions were separated by high-performance liquid chromatography and then identified by e.s.r. Deamination (ring-opening reaction for L-proline) was observed for all amino acids. For L-valine and L-leucine, H-abstraction from the tertiary carbon in the side chains occurred. For isoleucine, H-abstraction from the  $\alpha$ -carbon of the amino acid and from a non-terminal carbon in the side chain were found. 7. Photoionization of aromatic amino acids in aqueous solutions.

A spin-trapping and electron spin resonance study (with M.M. Mossoba and K. Makino). The wavelength dependence (280-334 nm) of the photoionization of tryptophan, tyrosine and phenylalanine in aqueous solution was investigated by means of e.s.r. and spin-trapping. Chloroethanol, glycine and alanine were used to scavenge the hydrated electrons in the pH range 7-10. The dechlorination radical from chloroethanol and the deamination radicals from glycine and alanine were spin-trapped with 2-methyl-2-nitrosopropane (MNP) and identified by e.s.r. From these observations it was inferred that photoionization occurred at  $280 \pm 10$  and  $313 \pm 7$  nm but not at  $334 \pm 10$  nm. No evidence for a photoionization threshold for tryptophan at 275 nm could be found. 8. Chemical effects of ultrasound on aqueous solutions. Evidence for the formation of  $\cdot\text{OH}$  and  $\cdot\text{H}$  in cavitation bubbles (with K. Makino and M.M. Mossoba). Direct evidence for the formation of  $\cdot\text{OH}$  and  $\cdot\text{H}$  from water by ultrasound was obtained. The method of spin-trapping with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), (4-N-methyl-pyridinium) tert. butyl nitron (PYBN) and  $\alpha$ -4-pyridyl-1-oxide N-tert-butyl-nitron (POBN) combined with e.s.r. was used for the detection of  $\cdot\text{OH}$  and  $\cdot\text{H}$ . With either DMPO or PYBN, the  $\text{OH}$ - and  $\text{H}$ - adducts were obtained, and with POBN the  $\text{H}$ -adduct was observed. These results were confirmed by sonolysis of  $\text{D}_2\text{O}$  solutions containing the same spin-traps. By studying the competition reactions between the spin traps, DMPO and POBN, and several  $\cdot\text{OH}$  and  $\cdot\text{H}$  scavengers it could be shown that the radicals originate in the bubbles produced by acoustic cavitation and not in the bulk of the solution. The implications of these results for the clinical use of ultrasound in therapy and diagnosis will be the subject of further investigations.

9. Identification on radicals in  $\gamma$ -irradiated aqueous solutions of proteins by e.s.r. and spin-trapping (with Alasdair Carmichael and F.H. White, Jr., Lab. of Cell Biology, NHLBI). A new method for the identification of radicals in polypeptides and polynucleotides is being developed. Since we have demonstrated that the radicals produced in  $\gamma$ -irradiated aqueous solutions of high molecular weight polypeptides and polynucleotides can be spin-trapped, it seems reasonable to expect that enzymatic hydrolysis or chemical degradation coupled with high pressure liquid chromatography, which permits the rapid separation of the constituent long-lived nitroxide radicals, will lead to the identification of radicals in proteins and nucleic acids. A similar approach could also be useful for studying the radicals produced when these macromolecules are irradiated in the solid state with  $\gamma$ -rays or with u.v.-light.

Significance to Cancer Research and the Program of the Institute: Studies of the effects of ionizing radiation are of importance in relation to (1) radiation therapy, (2) carcinogenesis, (3) stability of the genetic pool, (4) the suppression of the immune mechanism, and (5) aging. The effects of ionizing radiation on nucleic acids are being studied in order to understand the nature of radiobiological death in normal and tumor cells. The addition of radio-protective and radiosensitizing agents is being investigated so that a therapeutic advantage may be gained. Objective 6, Approach 1.



Proposed Course of Research: To continue studies on the effects of ionizing radiation on macromolecules of biological importance. The mechanism of radioprotective and radiosensitizing agents and the interaction of radiation and cancer chemotherapy agents will be investigated.

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Kuwabara, M., Lion, Y., and Riesz, P. E.s.r. of spin-trapped radicals in aqueous solutions of 5-halo derivatives of nucleic acid constituents: Reactions of hydrated electrons, hydroxyl radicals and UV photolysis. Int. J. Radiat. Biol. 39: 491-514, 1981.

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Rosenthal, I., Mossoba, M.M., and Riesz, P. Dibenzoylperoxide-induced photo-decarboxylation of amino acids and peptides. A spin-trapping study. J. Phys. Chem. 85: 2389-2403, 1981.

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Lion, Y., Kuwabara, M. and Riesz, P. Spin-trapping and e.s.r. studies of the direct photolysis of aromatic and amino acids, dipeptides, tripeptides and polypeptides in aqueous solutions. III. Tryptophan and related compounds. Photochem. Photobiol. 35: 53-62, 1982.

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Riesz, P. and Rosenthal, I. Photochemistry of protein and nucleic acid constituents: electron spin resonance and spin-trapping with 2-methyl-2-nitrosopropane. Can. J. Chem. 60: (in press).

Makino, K., Mossoba, M.M. and Riesz, P. Chemical effects of ultrasound on aqueous solutions. Evidence for  $\cdot\text{OH}$  and  $\cdot\text{H}$  by spin-trapping. J. Am. Chem. Soc. (in press).

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 00944-20 LPP
PERIOD COVERED <u>October 1, 1981 to September 30, 1982</u>		
TITLE OF PROJECT (80 characters or less) Total Metabolism of Cancer Cachexia		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Seoras D. Morrison      Research Physiologist      LPP, NCI		
COOPERATING UNITS (if any) Surgical Metabolism Section, Surgery Branch, NCI		
LAB/BRANCH <u>Laboratory of Pathophysiology</u>		
SECTION <u>Energy Metabolism Section</u>		
INSTITUTE AND LOCATION <u>NCI, NIH Bethesda, Maryland 20205</u>		
TOTAL MANYEARS: <u>2.5</u>	PROFESSIONAL: <u>1.0</u>	OTHER: <u>1.5</u>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  This project is directed towards identifying the causes of the <u>nutritional</u> <u>depletion</u> and general deterioration of the <u>host</u> , known as <u>cancer cachexia</u> , with a view to blocking or reversing these <u>systemic effects of cancer</u> so that the cancer patient could become more accessible to anti-cancer therapies. The approach to this is by investigation of <u>total energy</u> , <u>water</u> and other material exchanges, and of the physiological and behavioral <u>control of food and water</u> <u>intake</u> in normal and cancerous organisms.		

**Project Description:**

**Objectives:** (a) Investigation of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity with a view to finding how the energy expenditure, feeding and general behavior pattern are related to tumor growth, to finding the nature of the loads imposed by a tumor on its host, and to identifying the causes of cancer cachexia. (b) Investigation of relative changes in water and material exchanges in tissue compartments of the rat during imposed and induced changes in food and water intake and the relation of these to tumor induction and growth. (c) Examination of metabolic effects of total parenteral nutrition and chemotherapy in non-tumor-bearing rats and on the cachexia and decline of food intake induced by tumor growth. (d) Identification of functional sites and causes of breakdown of control of food intake during tumor growth. (e) Development of conceptual models of control of food and water intake and regulation of energy and water exchange and their inter-relationships for normal animals and for the cancer cachectic process.

**Methods Employed:** The methods of indirect, total, long-term calorimetry, operant conditional responses, placement of electrolytic lesions in or stimulation of the central nervous system, continuous or programmed infusions into unrestrained animals, and methods of orthodox nutritional studies. Computer methods of numerical analysis of serial records of tumor growth, continuous records of gaseous exchange and of change in feeding and drinking patterns in relation to total energy exchange and tumor growth. Sprague-Dawley rats, Buffalo rats and Fischer rats are used in all animal experiments. Most tumor-bearing rats are inoculated with Walker 256 carcinoma, but other transplantable tumors are also used.

**Major Findings:** Work is continuing on the behavioral and metabolic origins of the decline in food intake that is largely responsible for the cachectic decay accompanying tumor growth.

The feeding responses of tumor-bearing animals to stimuli that induce feeding response in normal animals are being examined. The feeding response to reduced environmental temperature is depressed by presence of tumor but the depression is independent of tumor burden at all reduced temperatures. The feeding response to exogenous insulin is enhanced by tumor growth: the reactive hypophagia and loss of bodyweight that normally occurs on withdrawal of insulin is attenuated in presence of tumor. This may represent a practicable procedure for combating cachectic anorexia.

A project on the effect of TPN in tumor-bearers in the absence of effective anti-tumor therapy shows that although this procedure can maintain host mass and composition, it increases rate of tumor growth and decreases survival time.

A project comparing the efficacy of enteral and parenteral feeding is continuing and has been expanded to examine effects of high lipid versus high carbohydrate aliment (collaborative project with Dr. James Shull, Surgery Branch) and to examine effects of continuous hyperalimentation of normal animals on motor activity. Relationships between satiety and sedation and discrimination between attenuated hunger and premature satiety as immediate mechanisms of cancer hypophagia are also being investigated.

Significance to Biomedical Research and the Program of the Institute: The findings on control of feeding in tumor growth are throwing light on the nature of cancer cachexia and should be utilizable in the development of effective methods for improving the nutritional condition of cancer patients. The section of the National Cancer Plan that the work most closely approximates is: Objective 6 (Develop the means to cure cancers and to retard the progress of cancers not cured). Approach 4 (Enhance the host's ability to eliminate or prevent further development of Cancer). It is also immediately relevant to the 1974 Amendment to the Cancer Act (Collect ....information respecting nutrition programs for cancer patients and the relationship between nutrition and cancer).

Proposed Course of Research: Work in energy and water exchange of tumor bearers will be continued along with study on the interaction of metabolic and behavioral responses of normal and cancerous animals, with particular reference to the reasons for the changes in food and water intake and depletion of host tissues that occur during tumor growth. Work is planned on the interaction of TPN and chemotherapeutic agents in influencing tumor growth and cachexia. Objective 6, Approach 4.

Publications:

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER 1CB 05211-10 LPP
PERIOD COVERED <u>October 1, 1981 to September 30, 1982</u>		
TITLE OF PROJECT (80 characters or less) Poly(ADP-ribose) and Chromatin structure and Function.		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: M.R. Purnell, Visiting Fellow LPP, NCI  Other: W.R. Kidwell Chief, Cell Cycle Regulation Section LPP, NCI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH <u>Laboratory of Pathophysiology</u> SECTION <u>Cell Cycle Regulation Section</u> INSTITUTE AND LOCATION <u>NCI, NIH Bethesda, Md. 20205</u>		
TOTAL MANYEARS: <u>1.0</u>	PROFESSIONAL: <u>1.0</u>	OTHER: <u>0</u>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <p> <u>Poly(ADP-ribose) synthetase</u> is a chromatin bound enzyme thought to be involved in some types of <u>DNA repair</u>. In intact cells the enzyme is inactivated by a non-lethal heat shock in both <u>Drosophila</u> and <u>Hela</u> cells. The repair of single strand DNA chain breaks was efficiently conducted in both cell types following <u>heat shock</u> indicating that this type of repair did not require synthetase action. Repair of this type of lesion was also not affected by synthetase inhibitors such as 3-aminobenzamide or 5-methylnicotinamide. The clonal growth of CHO cells was dramatically reduced by brief exposure to 3-acetylaminobenzamide, one of the most potent synthetase inhibitors known. 3-aminobenzamide also caused the death and resorption of mouse fetuses. Thus, the synthetase probably plays some roles in cells other than, or in addition to, DNA repair. This synthetase inhibitor had no effect on tyrosine transaminase induction in HTC cells nor on the methylation of deoxycytidine in DNA. Thus, there is no evidence for a role of the synthetase in <u>gene expression</u> in these cells.         </p>		



## Project Description:

Methods Employed: Single strand breaks were introduced into Hela or Drosophila cells by  $\gamma$ -irradiation. The extent of breakage or the rate of repair of breaks were assessed by alkaline sucrose gradient sedimentation or by the ethidium bromide intercalation method of Birnboim and Jevcak. Effects of heat shock or poly(ADP-ribose) synthetase inhibitors were assessed by comparing the clonal growth of control and treated cells.

Major Findings:

1. The synthetase activity is inactivated by heat shock of cells. With Drosophila cells, a 5 min. heat shock at 8°C above physiological temperature transiently inactivated all poly (ADP-ribose) synthetase activity but had no effect on the viability of the cells. In Hela cells, the synthetase was also found to be temperature sensitive. Incubating the cells for 15 min at 45° reduced the synthetase by about 50% while a 30 min heat shock reduced the enzyme by more than 90%. Cell viability was reduced by 5 and 25%, respectively by such treatments. The temperature sensitivity of the synthetase in Hela could be increased by briefly omitting nicotinamide or glutamine, factors required for substrate NAD synthesis, from the growth medium. Higher levels of nicotinamide or analogs of nicotinamide, such as 3-aminobenzamide, which bind to the NAD binding site on the enzyme, stabilize the synthetase against heat inactivation in intact cells.

2. DNA single strand break repair takes place efficiently in the absence of poly(ADP-ribose) synthesis. In heat shocked Drosophila cells in which no poly(ADP-ribose) synthetase remained, the repair of  $\gamma$ -ray induced single strand breaks in DNA was equal to that seen in non-heat shocked cells. Addition of the synthetase inhibitors, 5-methylnicotinamide or 3-aminobenzamide also did not slow the rate of repair of such lesions as measured by sedimentation of DNA on alkaline sucrose gradients. Even though the synthetase is activated by single strand breaks in DNA, apparently because of increased affinity for regions of DNA with chain breaks present, such lesions did not affect the temperature sensitivity of the synthetase in Drosophila. Poly(ADP-ribose) synthetase was also temperature sensitive in Hela cells. Utilizing the very sensitive Ethidium Bromide intercalation assay method of Birnboim and Jevcak, the repair of  $\gamma$ -ray induced DNA chain breaks was found to be unaffected by a heat inactivation of poly(ADP-ribose) synthetase or by blocking the synthetase with 3-aminobenzamide in Hela cells.

3. Poly(ADP-ribose) synthetase inhibitor effects on cell growth and differentiation. Although poly(ADP-ribose) synthetase may function in some types of DNA repair, the enzyme is probably also needed for cell growth. All inhibitors of the synthetase slow or arrest cell growth at high concentration. A new synthetase inhibitor, 3-acetylaminobenzamide was synthesized and found to be the most potent inhibitor of the enzyme tested to date. This compound also blocked the clonal growth of CHO cells at much lower concentrations than other synthetase inhibitors. The deacylated form of this compound also caused the death of mouse embryos in utero. Some reports have indicated that differentiation is



is induced or blocked by synthetase inhibitors. This possibility was examined using HTC cells. Tyrosine transaminase was inducible in these cells by Dexamethasone. Induction of transaminase was unaffected by 3-aminobenzamide. This compound also had no effect on the formation of methyldeoxycytidine which is purportedly decreased in cells induced to differentiate.

#### Significance to Biomedical Research and the Program of the Institute:

Poly(ADP-ribose) synthetase inhibitors have been shown to potentiate the killing of tumor cells by certain types of agents which damage DNA, suggesting that the enzyme is needed for repairing DNA lesions. The present report shows that single strand break repair is efficiently performed in the absence of a functioning synthetase, however. Other types of DNA lesion repair may involve synthetase action. Inhibitors of the synthetase may be of clinical importance for potentiating the killing of cancer cells by chemotherapeutic agents which produce such DNA lesions.

Proposed Course of Research: Our research for the coming year will focus on the role of the synthetase in the repair of DNA lesions produced by alkylating agents, DNA-DNA crosslinking and DNA-protein crosslinking agents. Both the heat shock and poly(ADP-ribose) synthetase inhibitor approaches will be utilized to determine possible synthetase involvement in these types of repair. The temperature sensitivity of the synthetase in Hela cells makes it unlikely that the synthetase is directly involved in hyperthermic killing of tumor cells. The increased thermal sensitivity of the synthetase in the absence of glutamine does suggest, however, that thermal inactivation may be achieved at lower temperatures by antimetabolites of glutamine such as azaserine and DON and by glutamine synthetase inhibitors. These possibilities will be evaluated. Additionally, a variety of new synthetase inhibitors will be synthesized and tested for their effects on DNA repair and cell growth.

#### Publications:

Kidwell, W.R., Nolan, N. and Stone, P.R.: Variations of poly(ADP-ribose) and poly(ADP-ribose) synthetase in synchronously dividing cells. In Hayaishi, O and Ueda, K. (Eds.): ADP-ribosylation Reactions, in press.

Nolan, N. and Kidwell, W.R.: Effect of heat shock on poly(ADP-ribose) synthetase and DNA repair in *Drosophila* cells. Radiation Res. 90: 187-203, 1982.

Kidwell, W.R.: Thermal sensitivity of poly(ADP-ribose) synthetase in Hela cells. In Sugimura, T. and Hayaishi, O. (Eds.): Poly(ADP-ribose) synthetase and DNA repair, Proceedings of the Princess Takamatsu Cancer Research Fund Conference, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)		U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER Z01 CB 05216-11 LPP	
PERIOD COVERED October 1, 1981 to September 30, 1982					
TITLE OF PROJECT (80 characters or less)  Cyclic Nucleotide-Growth Regulatory Mechanism					
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT					
PI: Y.S. Cho-Chung		Chief, Cellular Biochemistry		LPP, NCI	
		Section			
Others: F. Huang		Expert		LPP, NCI	
T. Clair		Chemist		LPP, NCI	
B. Berghoffer		Biologist		LPP, NCI	
J. Katz		Chemist		LPP, NCI	
C. Shepherd		Biologist		LPP, NCI	
COOPERATING UNITS (if any)					
LAB/BRANCH Laboratory of Pathophysiology					
SECTION Cellular Biochemistry Section					
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Md. 20205					
TOTAL MANYEARS: 4.5		PROFESSIONAL: 1.0		OTHER: 3.5	
CHECK APPROPRIATE BOX(ES)					
<input type="checkbox"/> (a) HUMAN SUBJECTS x		<input type="checkbox"/> (b) HUMAN TISSUES		<input type="checkbox"/> (c) NEITHER	
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS					
SUMMARY OF WORK (200 words or less - underline keywords)					
<p>During regression of <u>hormone-dependent mammary tumors</u> produced with a variety of procedures (<u>ovariectomy</u>, <u>DBcAMP</u>, <u>choleratoxin</u> or <u>antiestrogens</u>) the <u>cAMP receptor level</u> was enhanced and the <u>estrogen receptor level</u> was depressed in an antagonistic manner. Within 6 hrs post <u>ovariectomy</u> or <u>DBcAMP treatment</u>, the <u>in vitro synthesis of several polypeptides</u> instructed by <u>poly(A)<sup>+</sup>RNAs</u> of the tumors was either depressed or stimulated and the same new set of genes are expressed in both <u>ovariectomy-</u> and <u>DBcAMP-induced</u> regressing tumors. The same changes in the <u>translation products</u> were also demonstrable in the tumor slices preincubated with <u>cAMP in vitro</u>, mimicking the regressing tumors <u>in vivo</u> and this cAMP effect was abolished when <u>17<math>\beta</math>-estradiol</u> was simultaneously present with cAMP. Thus, mammary tumor growth is subject to <u>transcriptional control</u> and that the <u>antagonism between cAMP and estrogen</u> is involved in this genetic event.</p>					

## Project Description:

Methods Employed:

- i. Tumors: Primary, 7,12-dimethylbenz( $\alpha$ )anthracene (DMBA)-induced mammary carcinoma and transplantable MTW9, DMBA #1, MT13762, mammary carcinomas were used.
- ii. MCF-7 cells: The MCF-7 cells (Mason Research Institute) were grown in McCoy's 5A medium supplemented with bovine insulin, penicillin, streptomycin and fetal calf serum (10%) and + additives.
- iii. cAMP assay: cAMP was measured by the competitive protein-binding method of Gilman using cAMP assay kit of Amersham.
- iv. Protein kinase assay: the activity was determined by measurement of the incorporation of  $^{33}\text{P}$  from  $\gamma$ -labeled ATP into histone +  $10^{-6}\text{M}$  cAMP.
- v. Estrogen-binding assay: Estrogen binding was measured by the modification of the charcoal adsorption assay described originally by Korenman.
- vi. In vitro translation: Total poly A containing mRNA was isolated from tumors by the method of Deeley et al. In vitro translation systems of both rabbit reticulocyte lysate and wheat germ extract were used. Total translation products were analyzed by SDS-polyacrylamide gel electrophoresis.

Major Findings:I. Growth arrest and cAMP-dependent protein kinaseA. Growth arrest by Cholera toxin

1. Growth of 7,12-dimethylbenz( $\alpha$ )anthracene (DMBA)-induced mammary carcinoma in rat was arrested by daily s.c. injections of Cholera toxin, the exo-enterotoxin of the bacterium *Vibrio Cholerae*. At a dose of 2  $\mu\text{g}/200\text{g}$  rat, tumors regressed to 50% of their initial size within 2-3 weeks and 85% of tumors regressed completely within 4-6 weeks.
2. The same response to Cholera toxin was observed with other hormone-dependent mammary tumor, MTW9, but not with hormone-independent tumors, DMBA #1 and MT 13762. The latter result was consistent with the lack of response of these hormone-independent tumors to hormone-removal (ovariectomy) or DBcAMP treatment.
3. The growth inhibitory effect of Cholera toxin was dose-dependent and upon cessation of treatment tumors resumed growth; after complete regression, however, tumors did not reappear until 6 months after termination of the treatment.
4. The amount of Cholera toxin as high as 10  $\mu\text{g}/\text{day}/200\text{g}$  rat injected over 6 weeks period was not toxic to the animals and did not produce measurable amount of anti-cholera-toxin-antibody in the sera of animals.

5. Growth of human breast cancer cells (MCF-7) in culture was also inhibited by a daily supplement of cholera toxin. At a concentration of 100 ng/ml, the cell replication ceased completely within 2 days and 1 ng/ml exhibited 50% growth inhibition by day 2. The growth arrest was maintained as long as the treatment was continued but was reversed upon cessation of the treatment.
6. The growth arrest of MCF-7 cells by cholera toxin accompanied a striking change in cell morphology which was similar to that observed after DBcAMP and arginine treatment of MCF-7 cells; the cytoplasm of the treated cells was greatly enlarged without appreciable change in the size of the nuclei.

#### B. Cyclic AMP level and cAMP-dependent protein kinase

1. The growth inhibition by Cholera toxin was preceded by the increases in the cellular cAMP content and cAMP dependent protein kinase activity. Within 24 hr post cholera toxin injection, the cAMP level and cAMP-dependent protein kinase activity increased 3- and 4-fold over the control values, respectively.
2. Concomitant with the increases in cAMP-dependent protein kinase activity was a decrease of estrogen binding in the tumors; the estrogen binding activity decreased to 50% of the control value within 24 hr of cholera toxin treatment.
3. The cAMP content and cAMP-dependent protein kinase activity of MCF-7 cells also markedly increased upon cholera toxin treatment. At 3 days after cholera toxin treatment, the cAMP level and the kinase activity increased 40- and 10-fold over the control values, respectively. Concomitantly, estrogen binding activity decreased by 60% in the cytosol.

These results suggest that extinction of mammary cancer can be achieved by cholera toxin, an agent that stimulates intracellular cAMP system.

## II. Genomic Regulation of Mammary Tumor Growth

### A. Genetic transcription in growing and regressing mammary carcinoma

1. Poly(A)<sup>+</sup> containing RNAs isolated from growing and regressing DMBA tumors were translated in cell-free protein synthesizing systems and their translation products were compared by SDS-polyacrylamide gel electrophoresis. Within 6 hr post ovariectomy, the concentration of one protein band (MW, 20.5K) increased and those of two protein bands (MW, 22K and 35K) decreased in the regressing as compared to the growing tumors.
2. Strikingly the translated protein patterns of the regressing tumors were identical whether regression was induced by ovariectomy or DBcAMP treatment.



3. Injection of 17 $\beta$ -estradiol to ovariectomized host produced resumption of tumor growth and reversed the changes in the translated protein pattern observed during regression.
4. Autonomously growing DMBA tumors exhibited protein patterns markedly different from that of hormone-dependent DMBA tumors, and the patterns did not change after ovariectomy or DBcAMP treatment.

#### B. Genetic transcription in tumor slices in vitro

The changes in genetic transcription was also demonstrable in vitro. Slices from growing DMBA tumors were preincubated at 30°C with cAMP ( $10^{-7}$ M) and then isolated mRNAs were translated in vitro. The same translation protein pattern as that of the regressing tumors in vivo was induced. Moreover, this cAMP effect was abolished when 17 $\beta$ -estradiol ( $10^{-9}$ M) was present simultaneously with cAMP.

These results suggest that mammary tumor growth is subject to transcriptional control and that the antagonistic interrelation between cAMP and estrogen is involved in this genetic event.

Significance to Cancer Research and the Program of the Institute: (NCP Objective #6, Approach #3. These studies contribute to the understanding of the mechanism of cAMP action in the regulation of mammary tumor growth. Cholera toxin, a very specific pharmacological tool for raising intracellular cAMP level caused extinction of the growth of mammary tumors in rat and human breast cancer cells in culture. The antagonistic action between cAMP and estrogen at the nuclear level is suggested by the data of in vitro translation of poly(A)<sup>+</sup>RNAs. These results together with our previous studies suggest a therapeutic potential for cAMP in human breast cancer. The use of dibutyryl cyclic AMP may substitute for, or synergize the effects of, antiestrogens or some of the cytotoxic agents presently in use.

Proposed Course of Research: To extend the investigation on the mechanism of cAMP action in growth control, the following proposal is made:

1. Characterize the molecular form of cAMP receptor complex that enters nucleus by the use of site specific anti-cAMP-dependent protein kinase antibodies.
2. Assess the role of cAMP receptor protein in the modulation of genomic expression during mammary tumor regression.
3. Assess the relationship between nuclear protein phosphorylation, cAMP-dependent protein kinase and gene transcription.
4. Identify the specific mRNAs associated with the growth and regression of mammary tumors.



Publications:

Cho-Chung, Y.S.: Mode of cyclic AMP action in growth control. In Leung, B.S. (Ed.): Hormonal Regulation of Experimental Mammary Tumors. Pergamon Press, in press.

Cho-Chung, Y.S.: Intracellular mediators of cancer growth and metastasis. In Stoll, B.A. (Ed.): Prolonged Arrest of Cancer. John Wiley & Sons Ltd, England, in press.

Cho-Chung, Y.S., Clair, T., Schwimmer, M., Steinberg, L., Rego, J.A. and Grantham, F.: Cyclic adenosine 3',5'-monophosphate receptor proteins in hormone-dependent and -independent rat mammary tumors. Cancer Res. 41: 1840-1846, 1981.

Cho-Chung, Y.S., Clair, T., Bodwin, J.S. and Berghoffer, B.: Growth arrest and morphological change of human breast cancer cells by dibutyryl cyclic AMP and L-arginine. Science 214: 77-79, 1981.

Bodwin, J.S., Hirayama, P.H. and Cho-Chung, Y.S.: Cyclic AMP-binding protein and estrogen receptor: antagonism during nuclear translocation in a hormone-dependent mammary tumor. Biochem. Biophys. Res. Commun. 103: 1349-1355, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  ZO1 CB 05219-11 LPP									
PERIOD COVERED October 1, 1981 to September 30, 1982											
TITLE OF PROJECT (80 characters or less)  In vitro Simulation of Hormone-dependent Mammary Tumor Regression											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: R.A. Knazek</td> <td style="width: 33%;">Senior Investigator</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>S.C. Liu</td> <td>Chemist</td> <td>LPP, NCI</td> </tr> <tr> <td>J.R. Dave</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> </table>			PI: R.A. Knazek	Senior Investigator	LPP, NCI	S.C. Liu	Chemist	LPP, NCI	J.R. Dave	Visiting Fellow	LPP, NCI
PI: R.A. Knazek	Senior Investigator	LPP, NCI									
S.C. Liu	Chemist	LPP, NCI									
J.R. Dave	Visiting Fellow	LPP, NCI									
COOPERATING UNITS (if any)											
LAB/BRANCH Laboratory of Pathophysiology											
SECTION Cell Cycle Regulation											
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 2.25	PROFESSIONAL: 1.50	OTHER: 0.75									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords) Alterations of the <u>hormone-receptors</u> on or within cells will modify the response of target tissues to various hormones thus serving to control cellular growth or function. We have shown that the number of detectable prolactin receptors is controlled <u>in vivo</u> by the circulating levels of prolactin or growth hormone by a positive feedback mechanism. Inhibition of <u>in vivo prostaglandin</u> (PG) synthesis by either enzymatic blockade or precursor depletion results in a loss of existing PRL receptors and prevents their induction by PRL. As membrane fluidity increases in a variety of hormonal, dietary or developmental states, the number of PRL receptors also increases. It has been demonstrated that PRL alters PG synthesis <u>in vivo</u> . These data show that PRL up-regulates its own receptor by modifying target <u>membrane fluidity</u> and that this may occur through modification of prostaglandin synthesis. Extended to the DMBA rat <u>mammary tumor</u> , the regressing tumor membranes are more viscous and bind less PRL than those of the growing tumor. An assay for PG receptor has been developed, which has demonstrated both that these regressing tumors have an increased capacity to bind PG and that copper increases this binding capacity 8-fold. Copper may, thus augment the effect of prostaglandins <u>in vivo</u> and play a role in tumor growth.											

## Project Description:

Objectives: Reproduce and study in vitro, the hormone-dependent regression process of mammary tumors in vivo.

Methods Employed: A radioreceptor assay for prolactin and growth hormone receptors is used to determine the ability of cell membranes to bind these polypeptide hormones.

Assay techniques for prostaglandin E and  $F_{2\alpha}$  have been devised consisting of extraction from incubation buffers by organic solvents, silicic acid double column chromatography, and specific radioimmunoassay. High pressure liquid chromatography is used to quantitate prostaglandin intermediates synthesized by various tissues.

Cell membrane fluidity was determined by fluorescence polarization. This was accomplished by measuring the ease of rotation of the fluorescent lipid probe diphenylhexatriene (DPH), that had been inserted into the membranes in vitro. The polarization constant was then translated mathematically into viscosity.

A method for the determination of the number and affinity of prostaglandin receptors was developed as follows: Either DMBA - or NMU-induced mammary carcinoma was excised from female Sprague-Dawley or Buffalo rats, respectively. The tumors were minced and then homogenized in 250 mM Sucrose, 10 mM Tris, 0.1% Na azide, 1 mM dithiothreitol, and 1 mM  $CaCl_2$ . After a preliminary low speed spin, the supernatant was centrifuged at 15,000 x g, and the resultant membrane pellet was used for binding studies. The membrane fractions were incubated at 15°C for 30 minutes with graded concentrations of [ $^3H$ ] PG in a total volume of 250  $\mu$ l which included 100  $\mu$ l of membrane suspension. After incubation was complete, a 125  $\mu$ l aliquot was filtered rapidly through a Whatman GF/C glass microfibre filter disc and washed with ice cold Tris buffer. The amount of PG bound to the membranes was determined by counting the air-dried filter in a scintillation counter.

Major Findings: PRL receptors can be induced in the rodent liver by in vivo injections of either purified bGH or bPRL. GH was shown to induce but not to bind to the receptor whereas PRL did both. These studies, plus the observation that induction occurred even if protein synthesis had been blocked, indicated that the induction was being achieved by exposing or altering receptor in such a way that its binding site was made available to PRL. Increases in the number of PRL binding sites could also be achieved by direct alteration of the phospholipid bilayer. This was accomplished both in vitro and in vivo. The PRL binding capacity increased by 60% when hepatocytes were incubated with either phospholipase  $A_2$  or lysolecithin but decreased by 60% when C3H mice were depleted of essential fatty acids by dietary means, a loss that could not be reversed by treatment with exogenous PRL. Blockade of prostaglandin synthesis by in vivo treatment with indomethacin also reduced existing hepatic-PRL receptors in a dose-responsive fashion that, again, could not be overcome by exogenous PRL injections. Modification of the hepatic prolactin receptor could, therefore, be achieved by changes in the character of the lipid bilayer, an effect related both to the ease with which pre-existing PRL receptors could assume an active configuration within

the lipid bilayer, i.e., the viscosity of the membranes could modify the activity of the receptor. Since the prolactin induces its own receptor *in vivo*, studies were undertaken to determine if this could, in fact, be a result of changes in membrane fluidity with concomitant exposure of pre-existing receptors. Injection of exogenous PRL in dosage schedules designed to achieve physiologic PRL levels in hypophysectomized rats did, indeed, serve to increase the phospholipid/cholesterol ratio by 24%, the membrane fluidity by 7%, and prolactin binding capacity 3-fold, whereas injection of supra-physiologic amounts of PRL reversed the effect. Measurement of the capacity of these hepatic membranes to synthesize PG revealed that physiologic replacement doses of PRL injected into either male or female rats caused an increase in the synthesis of PGF<sub>2</sub> $\alpha$  and PGE as well as membrane fluidity.

These data indicate that PRL modifies both the viscosity and PG synthesis by hepatic membranes *in vivo* and that these phenomena, together, might be responsible for the auto-regulation of detectable PRL receptors.

Additional studies demonstrated that incubation of hepatic membranes with PG1<sub>2</sub> *in vitro* resulted in an increase in both membrane fluidity and the number of prolactin receptor sites. Further experiments showed that *in vitro* incubations with phospholipase A<sub>2</sub>, arachidonic acid, or bradykinin, all participants in the early portion of the PG cascade, served to increase membrane fluidity and prolactin binding. In fact, any treatment of the hepatic membranes that increased membrane fluidity also resulted in increased prolactin binding. This was also shown in developmental studies wherein PRL receptors appeared in murine hepatic membranes at 21 days of age, a time when the membranes were also shown to be the most fluid of any developmental stage. Similar observations hold in the pregnant mouse: both PRL binding and hepatic membrane fluidity are greatest at days 12-16 of gestation. Prolactin binding levels and fluidity were not significantly different between the non-pregnant, non-lactating and the lactating group. Again, membrane fluidity was proportional to the phospholipid/cholesterol ratio.

The concept of a correlation between PRL binding and membrane fluidity was extended to those hormone-dependent rat mammary tumors induced by DMBA and NMU. When the hosts bearing these tumors are oophorectomized, serum levels of PRL fall, the numbers of tumor-associated PRL receptors decrease, and the tumors regress.

As predicted by the studies of liver, the tumor membranes were significantly more viscous after oophorectomy and possessed only a small percentage of the original number of prolactin receptors. The rates of PGE and F<sub>2</sub> $\alpha$  synthesis rose from 0.13 and 10.5 ng/mg protein in growing tumors to a maximum of 1.2 and 26.5 ng/mg protein by 5 days after oophorectomy. Thus, the apparent role of prostaglandins in the modulation of cell membrane fluidity with resultant changes in plasma membrane receptors, suggests that cellular response to prostaglandins might be controlled further by alterations in their ability to respond to various prostaglandins. To this end, a prostaglandin-receptor assay was devised wherein mitochondrial membranes were obtained from DMBA and NMU tumors, incubated with <sup>3</sup>H-PG (E<sub>2</sub> or F<sub>2</sub> $\alpha$ )  $\pm$  excess unlabeled PG. The bound and free PG were separated using the filtration technique developed as above. Binding of both PGE<sub>2</sub> and PGF<sub>2</sub> $\alpha$  to tumor membranes reached equilibrium after 30 minutes incubation. The binding was 44-58% reversible



within 15 minutes of adding excess unlabeled  $\text{PGE}_2$  or  $\text{F}_2\alpha$ , respectively. Specific binding of both types of PG increased 2-3 fold by ~ 5 days after oophorectomy. The  $K_d = 2.9 \text{ nM}$  for  $\text{PGF}_2\alpha$  binding to growing tumor membranes from intact hosts with a binding capacity of 25 fmole/mg protein. An increase in binding capacity was noted after oophorectomy while  $K_d$  remained unchanged. Only a very low affinity  $\text{PGE}_2$  receptor was detectable in both growing and regressing tumor membranes.

In view of our previous studies on the hormonal control of mammalian follicular maturation and oogenesis (Z01-CB-08230-06 LPP), we suspected that prostaglandins might be playing a role in stimulating the formation of new blood vessels within the tumor. This was supported by the studies of Ziche and Gullino that showed PGE was angiogenic in the rabbit cornea. Furthermore, they demonstrated that copper was also angiogenic. This prompted us to study the effect of copper on  $\text{PGE}_1$  binding to tumor membranes.

Addition of graded doses of  $\text{CuCl}_2$  to the prostaglandin binding assay resulted in an 8-fold increase in PG binding, reflecting the appearance of a high affinity receptor. Neither  $\text{CaCl}_2$  nor  $\text{MgCl}_2$  were able to achieve the same effect. Similar increases in PG binding to liver membranes were induced by the addition of  $\text{Cu}^{++}$  to the incubation mixture. The data presented herein suggest that copper may play an obligatory role in neovascularization by increasing the number of binding sites for the angiogenic prostaglandins.

Significance to Biomedical Research and the Program of the Institute: Demonstration that prolactin receptors are induced by growth hormone indicates that this hormone may play an important role in the responsiveness of mammary carcinoma to hormonal stimuli. The fact that changes in membrane fluidity are induced by prolactin may help to explain the mechanism by which prolactin induces its own receptor. The requirement for essential fatty acids and prostaglandins to modulate the prolactin receptors indicates that the lipid stores play an important role in maintaining the responsivity of target tissues to circulating hormones. These studies support and explain, in part, the demographic correlation of mammary carcinoma with high dietary fat intake throughout the world. The importance of prostaglandins in the growth and metastasis of mammary tumors has been suggested by many investigators. Changes in their rates of production and ability to bind to tumor membranes as a function of hormonal status strengthens this hypothesis and suggests another mechanism by which tumor growth and metastases may be modified.

Proposed Course of Research: The role of prostaglandins in the induction of the prolactin receptor and prolactin action will continue to be studied in vivo and in vitro. The role of prostaglandins in the development of the mammary tumors and mammary glands will also continue to be studied using radioimmunoassay, radioreceptor, and HPLC techniques.

#### Publications:

Knazek, R.A. and Liu, S.C.: The effects of dietary essential fatty acids on murine mammary gland development. Cancer Research 41: 3750-1, 1981.



Onedera, T., Toniolo, A., Ray, U.R., Jenson, A.B., Knazek, R.A., Notkins, A.L.: Virus-induced diabetes mellitus. II. Polyendocrinopathy and autoimmunity. J. Exptl. Med. 153: 1457-1473, 1981.

Knazek, R.A., Liu, S.C., Dave, J.R., Christy, R.J., Keller, J.A.: Indomethacin causes a simultaneous decrease of both prolactin binding and fluidity of mouse liver membranes. Prostaglandins and Medicine 6: 403-411, 1981.

Dave, J.R., Knazek, R.A., Liu, S.C.: Prolactin modifies the fluidity of rat liver membranes. Biochem. Biophys. Res. Commun. 100: 45-51, 1981.

Liu, S.C., Knazek, R.A.: Prostaglandin synthesis and binding by growing and regressing rat mammary carcinoma. In: Proceedings of the International Conference on Prostaglandins and Cancer, Washington, D.C., 1981, Alan R. Liss, Inc., N.Y.

Dave, J.R. and Knazek, R.A.: Arachidonic acid, bradykinin, and phospholipase A<sub>2</sub> modify both prolactin binding capacity and fluidity of mouse hepatic membrane. Biochem. Biophys. Res. Commun. 103: 727-738, 1981.

Kidwell, W.R., Knazek, R.A., Vonderhaar, B.K., Losonczy, I.: Effects of unsaturated fatty acids on the development and proliferation of normal and neoplastic breast epithelium. In M.S. Arnott, J. van Eys, Y.-M. Wang (Eds.): Molecular Interrelations of Nutrition and Cancer Raven Press, N.Y. 1982. pp. 219-236.

Liu, S.C. and Knazek, R.A.: PG synthesis and binding is increased in regressing NMU-mammary carcinomas. Prostaglandins and Medicine (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08205-11 LPP
PERIOD COVERED <b>October 1, 1981 to September 30, 1982</b>		
TITLE OF PROJECT (80 characters or less) <b>Application of Artificial Capillary Culture Technique to Hormone Production</b>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: <b>R. A. Knazek</b> Others: <b>P. M. Gullino</b> <b>R. S. Balaban</b>	<b>Senior Investigator</b> <b>Chief</b> <b>Staff Fellow</b>	<b>LPP, NCI</b> <b>LPP, NCI</b> <b>LKEM, NIH, BLD</b>
COOPERATING UNITS (if any)		
LAB/BRANCH <b>Laboratory of Pathophysiology</b>		
SECTION <b>Cell Cycle Regulation Section</b>		
INSTITUTE AND LOCATION <b>NCI, NIH, Bethesda, Maryland 20205</b>		
TOTAL MANYEARS: <b>0.25</b>	PROFESSIONAL: <b>0.25</b>	OTHER: <b>0</b>
CHECK APPROPRIATE BOX(ES)		
<div style="display: flex; justify-content: space-around;"> <span><input type="checkbox"/> (a) HUMAN SUBJECTS</span> <span><input type="checkbox"/> (b) HUMAN TISSUES</span> <span><input checked="" type="checkbox"/> (c) NEITHER</span> </div> <div style="display: flex; justify-content: center; margin-top: 5px;"> <span><input type="checkbox"/> (a1) MINORS</span> <span><input type="checkbox"/> (a2) INTERVIEWS</span> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>           The <u>artificial capillary culture</u> technique provides a pericellular microenvironment that closely resembles the <u>in vivo</u> state. This may allow normal cells to maintain their differentiated functions for prolonged periods of time <u>in vitro</u>. Cells are maintained in a physiologic state without many of the artifactual conditions imposed by more standard methods of <u>tissue culture</u>. The technique is, therefore, especially well suited for the culture of cells both of endocrine origin and their target cells. Response of cells to various hormones and their culture over long periods have been achieved thus making the techniques useful for continuous production of hormones for both laboratory and clinical uses. Modifications have been made to the basic design. These permit study of nutrient and product transport through the tissues formed in the intercapillary spaces, the effect of lymphatic-type drainage upon cell function, effects of a physiologic collagen sub-stratum upon cell growth, and study of cell physiology by nuclear magnetic resonance.         </p>		

Project Description:

Objectives: A method is being developed for studying hormonally responsive tissues in vitro. Mammary, ovarian, and renal tissues are being used to study the mechanisms of hormonal stimulation.

Methods Employed: A bundle of tube-shaped semi-permeable membranes perfused with tissue culture medium, simulates the in vivo capillary bed. Isolated cells or tissue masses injected into the extracapillary space receive nutrients and have cell products removed by diffusion through the capillary walls. In this nearly physiologic environment, established cell lines form solid masses of tumor while primary tissue explants continue to function in a normal manner, in some cases, for several months. Fashioned into a "U" shape, the culture unit has been inserted into an NMR probe and perfused with culture medium and thus maintained at near-physiologic conditions while using  $^{31}\text{P}$  NMR to study intracellular metabolites.

Major Findings: The standard artificial capillary culture unit, which was developed in this laboratory, has previously been shown to provide a nutrient matrix which permits established cells to grow and form solid tumor masses in vitro. Secretory cells have retained their function in such a system for several months. Modification of the recirculating perfusion medium into a single pass mode of operation has demonstrated its value in studying the response of cell masses to physiologic hormones and chemotherapeutic agents.

Two separate capillary bundles were woven together and placed in a plastic shell fitted with Y connectors at each end. This provided matrices that were simultaneously intimately intertwined but still distinctly separate. Operation of the same or different hydrostatic pressures in co-current or counter-current perfusion modes permitted study of transport of solutes by diffusion or bulk flow through tissues grown within this braided network. The culture unit has been modified by wrapping the fiber bundle with a porous polymeric sheet which permits cells and particulate cell products to migrate or diffuse away from the cell mass. This permits harvesting of these substances without gross disturbance of the cell culture.

NMR investigation of HTC<sub>19</sub> cells and human lymphocytes have been initiated, studying both intracellular pH and energy metabolism. A6 cells have grown within the capillary culture device and preliminary  $^{31}\text{P}$  NMR spectra have been collected.

Significance to Biomedical Research and the Program of the Institute: The characteristics of the capillary culture unit enable rapid responses to both stimuli and inhibitors of cell function to be studied in a nearly physiologic in vitro environment. This work has demonstrated that convection as well as diffusion of cell nutrients and products plays a role in the transport of various substances within tumors. This may be of assistance in pharmacokinetic modeling and in understanding and improving the transport of both physiologic and pharmacologic agents within solid tumors. The study of cell behavior using NMR techniques may now be possible and provide a unique opportunity to study tumor physiology by a non-invasive method.

Proposed Course of Research: Ovarian, mammary tissues, and hormone-producing transformed cells will be studied within the artificial capillary culture unit in conjunction with our efforts to study the growth and regression of hormonally-responsive tumors in vitro. NMR studies will be made on high density cultures, studying the effects of hormonal, nutrient, and drug manipulations.

Publications:

Knazek, R.A. and Gullino, P.M. Cell culture on semi-permeable tubular membranes encased in a porous envelope. U.S. Patent 4,220,725.





Project Description:

Four areas have been emphasized during this year:

1) Continuation of previous work on the induction of synthesis of specific proteins in human lymphocytes upon exposure to interferon. 2) Characterization of a unique post-translational modification (hypusine formation) which occurs in a single protein in lymphocytes and other growing cells. 3) Examination of the synthesis of major histocompatibility antigens (HLA) in resting and growing lymphocytes and their relationship to cellular interactions of a proliferative and non-proliferative nature. 4) Studies of cell differentiation induced in cultured cell lines when exposed to phorbol esters.

Methods Employed: Human peripheral lymphocytes were prepared from heparinized venous blood of normal donors, purified by density sedimentation and nylon column adsorption. HL-60 cell line was carried under standard cell culture conditions. Two dimensional gel electrophoresis was carried out by the method of O'Farrell and analyzed by staining either with Coomassie blue or silver stain, and fluorography for detection of radiolabelled proteins.

Protein synthesis rates were measured by incorporation of [ $^3\text{H}$ ] leucine into acid insoluble material. DNA was measured by the Burton procedure; protein was quantitated by the Bradford procedure.

Chemotaxis was measured by migration through nucleopore membranes in Boyden chambers.

Protein purifications were carried out by gel filtration, DEAE-cellulose chromatography, and recovery from 2-dimensional gels.

Cell fractionations were carried out by various detergent-lysis techniques, or by sonication in sucrose solutions followed by differential centrifugation.

Identification of specific proteins was performed by exposing appropriate cell fractions to monoclonal antibodies, followed by binding of antigen-antibody complexes to protein-A-bearing fixed staphylococci. Specific proteins were recovered by centrifugation of staphylococci and elution with 9M urea, 2% NP-40, 5% 2-mercaptoethanol at 37°.

Major Findings: 1) We previously observed that partially purified interferon (IFN) preparations induced the synthesis of 8 specific proteins (I-peptides) in lymphocytes from all normal donors. These proteins were apparently not involved in maintenance of the low rate of protein synthesis characteristic of resting lymphocytes. This study was extended by the use of pure IFN produced by genetically manipulated bacteria (gift of Dr. Charles Weissmann, Zurich). The same 8 I-peptides were found to be induced, but there was greater variability in the responsiveness of different cell donors to the purified material as compared with partially purified IFN from natural sources. This may be explained by the fact that the cloned IFN was the product of only one of at least 8 different IFN genes, each encoding a slightly different IFN molecule, all of which are present in the IFN mixture produced by virus infected cells. Thus, different normal donors may exhibit genetic variation in the presumptive binding site for IFN, favoring one or another of the components of the natural mixture. Providing

only a single component as in treatment with cloned IFN, may reveal this variability.

The specific induction of 8 recognizable peptides furnishes a sensitive biochemical test which is useful in two ways. First, it may be considered a screening procedure for new presumptive IFN preparations, especially those which may be produced by recombinant DNA methodology where the completeness of the genetic information may be uncertain. Added to viral protection, this highly specific cell response increases the certainty that a given IFN preparation retains all the activities of the naturally occurring material. Second, this response may be used to determine the level of sensitivity of the cells of subjects who are prospective recipients of IFN for clinical purposes. Our studies showed that different normal donors require different IFN concentrations for maximal I-peptide induction. It is of obvious interest to determine whether this variable sensitivity may be related to clinical responsiveness at a given dose. In order to perform clinical screening, we have modified our technique so that a given donor can be screened for I-peptide responsiveness to 2 concentrations of IFN with a 30 ml. sample of heparinized blood.

In collaboration with the Frederick Cancer Research Center (Dr. Sherwin), which was about to begin phase II clinical trials of cloned, bacterially produced IFN- $\alpha_2$ , we have screened 19 successive patients with various neoplasms (predominantly advanced mammary carcinoma). Each donor's lymphocytes were exposed to either 10 or 100 units/ml. of IFN- $\alpha_2$  and the I-peptide response was determined by 2-dimensional gel electrophoresis. At this writing all of the preliminary analyses are complete. The fluorographs will now be quantitated by computerized densitometry and the enhancement of synthesis of each I-peptide will be determined. This data will then be correlated with the clinical results to determine whether I-peptide response serves in any way as a predictor of clinical response.

Additional studies with this system will be directed at identifying the I-peptides or equating them with known enzymatic activities induced by IFN. Studies on subcellular localization will also be pursued.

2) In collaboration with the Enzyme Chemistry Section, NIDR, we have discovered a novel post-translational modification of a single protein in lymphocytes and other growing cells. To date, our information on this modification is as follows:

A site-specific enzyme system recognizes a single lysine-containing region of a specific cytosolic protein of  $M_r \sim 17$  kD and  $pI \sim 5.1$ . We have termed this peptide Hy. To the  $\epsilon$ -NH<sub>2</sub> terminus of this lysine a 4-carbon segment derived from spermidine is covalently bound. Subsequently, a hydroxyl group is added to the 2-carbon of this added group. The modified lysine so produced [ $N^\epsilon$  (4-amino-2-hydroxybutyl)lysine] was named hypusine by its original discoverers.

The Hy protein is continuously synthesized by resting and growing lymphocytes. However, only after growth stimulation does the hypusine modification occur. The enzyme system performing this modification begins to be active after about 4 hrs. of growth and reaches a maximum about 24 hrs. after growth stimulation. Activation of this enzyme system is therefore one of the moderately early biochemical changes

associated with lymphocyte growth stimulation. While the role of this protein and of the hypusine modification are currently unknown, their importance is indicated by the fact that the same modification, of an apparently identical peptide, occurs in the growing cells of every cell type (6 cell types, 3 species) thus far examined. Moreover, the Hy protein of human and hamster cells showed identical electrophoretic mobility in 2 dimensions, suggesting a high degree of conservation of the structure of this protein throughout evolution and indicating strong selective pressure for its retention.

This study is being continued, as follows: Partial purification of Hy by column chromatography and 2-dimensional electrophoresis has been performed. This material is being used to produce a monoclonal antibody which will be used in large scale purification of both the hypusine-modified and the unmodified forms of Hy. This material will be assayed for effects it may have in various whole-cell and cell-free systems- e.g. - translation, transcription, membrane transport, in an effort to assign a general role to Hy. The monoclonal antibody will also be used to select polyribosomes carrying the mRNA for Hy with a view to preparing DNA probes and recombinant DNA reagents for further characterization of this unique protein and its gene structure.

3) HLA proteins are important glycoproteins of the plasma membrane of human lymphocytes and other cells providing the major histocompatibility determinants of human tissues. Although important in cellular immunological responses, their presence, production, and function in unstimulated lymphocytes has been little investigated. Examination of the proteins synthesized by resting lymphocytes by 2-dimensional electrophoresis revealed prominent synthesis of a group of peptides of  $M_r \sim 43$  kD and pI ranging from  $\sim 5.6$  to  $6.4$ . Another prominently synthesized peptide of  $M_r \sim 12$  kD and pI  $\sim 6.8$  was also seen. By immunoprecipitation with monoclonal or high titer polyclonal antibodies, these proteins were shown to be, respectively, the polymorphic HLA - A,B,C heavy chain ( $M_r - 43$  kD) and its accompanying light chain,  $\beta$ -2 microglobulin. By cell fractionation it was confirmed that these were primarily restricted to the cell membrane. By simple inspection it was evident that a major portion of the protein synthetic activity of resting lymphocytes was devoted to continuous turnover of these membrane proteins, in the absence of immunological or mitogenic stimulation. Upon growth stimulation with the non-specific mitogen, phytohemagglutinin, the synthesis of all proteins is enhanced many-fold. The synthesis of HLA and  $\beta$ -2 microglobulin, however, was evidently enhanced to a lesser degree than most other proteins. Thus, it appears that continuous turnover of membrane HLA may be of greater significance for resting than growing lymphocytes. We are currently quantitating these changes by means of computer-assisted densitometry. These studies will be extended to purified lymphocyte subpopulations, to determine whether these activities are characteristic of T or B lymphocytes, or both. By a modification of our 2-dimensional electrophoretic technique, we have obtained a marked increase in resolution of the polymorphic HLA proteins, and it appears that the resulting pattern is highly specific for a given individual. It is possible that this may provide an aid to histocompatibility typing. Specific sera for such HLA typing are available for a limited range of these proteins, while the 2-dimensional method visualizes all the peptides of the complex in a "fingerprint" fashion.



The significance of the rapid turnover of resting lymphocyte HLA is unknown, but we have formulated a hypothesis relating this phenomenon to immunological recognition and self-tolerance. Studies based on this hypothesis are being prepared.

4) Treatment of a human promyelocytic cell line (HL-60) with phorbol-12-myristate-3-acetate (PMA) for 48 h. induces terminal differentiation of these cells into monocytes. The effect of PMA involves arrest of growth, induction of cell adherence and development of esterase activity. Using Boyden chambers, we found that the cells show a high degree of spontaneous motility after PMA-induced differentiation.

Using 2-dimensional electrophoresis we have examined the particular proteins whose synthesis may be induced or modified as part of the differentiation process. A number of proteins were detected whose synthesis increased reproducibly upon PMA treatment and differentiation. Upon cell fractionation these proteins were found to be restricted to the membrane fraction, with little discernible effect on the cytosol. Comparison of these results with the 2-dimensional protein distributions of freshly prepared human monocytes revealed that the induced proteins were characteristically produced in large amounts by monocytes, while they were absent from peripheral lymphocytes thereby demonstrating the relevance of these proteins to the specific monocyte differentiation pathway. Thus, specific biochemical concomitants of the differentiation process have been identified, in addition to the functional and morphologic characteristics generally cited.

The differentiation process was also associated with a doubling of carboxy-methylation. A drug which is known to inhibit transmethylation, 3-deazoadenosine (DZA) markedly inhibited the induction by PMA of cell adherence and motility. Studies are in progress to determine the effect of DZA on specific protein induction in order to further understand the role of these proteins in the differentiation process.

5) In addition to the preceding studies, in collaboration with Dr. Roberto Fagnani, now at the U. of California, a one-step purification of T-cell growth factor (Interleukin-2) has been devised. Work on this technique is nearly complete.

Significance to Biomedical Research and the Program of the Institute: The work described is aimed at an understanding of the biochemical mechanisms underlying the regulation of synthesis and post-translational modification of proteins and hence, of cell growth, in normal human cells under physiological control as they respond to mitogenic or other modulating stimuli. In addition, the proteins involved in cell to cell interaction and recognition are under examination. Together, these form a contribution to the basic biochemistry of normal cell growth control, which is an essential component of basic cancer research.

In addition, where applicable, clinical extension of our observations (as in the I-peptide study) is undertaken in an effort to bring results in the laboratory into the clinical area in an expeditious manner. This is a major commitment of the NCI program.

Proposed Course of Research: As indicated under individual sections above.

Publications:

Park, M.H., Cooper, H.L., and Folk, J.E.: Identification of hypusine predominantly in a single protein of human lymphocytes: Spermidine as its precursor. Proc. Natl. Acad. Sci. USA 78: 2869-2873, 1981.

Resch, K., Wood, T., Northoff, H. and Cooper, H.L.: Microtubules: are they involved in the initiation of lymphocyte activation? Eur. J. Biochem. 115: 659-664, 1981.

Cooper, H. L. and Braverman, R.: Close correlation between initiator methionyl tRNA level and rate of protein synthesis during human lymphocyte growth cycle. J. Biol. Chem. 256: 7467-7467, 1981.

Cooper, H.L., Fagnani, R., London, J. and Trepel, J. Effect of interferon on protein synthesis during lymphocyte activation. In Kirchner, H. and Resch, K. (Eds.): Mechanism of lymphocyte activation, Elsevier, Amsterdam, 1981, pp. 179-191.

Cooper, H., Fagnani, R., London, J., Trepel, J. and Lester, E.: Effect of interferons on protein synthesis in human lymphocytes: enhanced synthesis of 8 specific peptides and activation-dependent inhibition of overall protein synthesis. J. Immunol. 128: 828-833, 1982.

Cooper, H.L.: Effect of bacterially produced interferon  $\alpha_2$  on synthesis of specific peptides in human peripheral lymphocytes. FEBS Lett. 140: 109-112, 1982.

Park, M.H., Cooper, H.L. and Folk, J.E.: Chromatographic identification of hypusine [ $N^{\epsilon}$ -(4-amino-2-hydroxybutyl)lysine] and deoxyhypusine [ $N^{\epsilon}$ -aminobutyl) lysine]. Methods in Enzymology, in press.

Cooper, H.L., Park, M.H., and Folk, J.E.: Post-translational formation of hypusine in a single major protein occurs generally in growing cells and is associated with activation of lymphocyte growth. Cell, in press.

Park, M.H., Cooper, H. and Folk, J.E.: The biosynthesis of protein bound hypusine ( $N^{\epsilon}$ -(4-amino-2 hydroxybutyl lysine): Lysine as the amino acid precursor and the intermediate role of deoxyhypusine ( $N^{\epsilon}$ -(4 aminobutyl) lysine). J. Biol. Chem., in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08212-08 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) From Gene to Protein: Structure, Function and Control in Eukaryotic Cells		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: S.L. Berger Others: D.M. Wallace R.S. Puskas	Research Chemist Visiting Fellow Senior Staff Fellow	LPP, NCI LPP, NCI LPP, NCI
COOPERATING UNITS (if any)		
Genentech, Inc. LAB/BRANCH Laboratory of Pathophysiology SECTION Office of the Chief INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.5	PROFESSIONAL: 2.5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
A <u>cdNA</u> sequence coding for <u>human immune interferon</u> has been identified in a <u>cdNA</u> library prepared from gel-fractionated <u>messenger RNA</u> obtained from stimulated lymphocytes. The DNA sequence codes for a polypeptide of 166 amino acids, 20 of which could constitute a signal sequence. The polypeptide produced by expressing this DNA sequence in <u>Escherichia coli</u> or cultured monkey cells has properties characteristic of authentic $\gamma$ -interferon.		

## Project Description:

Major Findings: Interferons are proteins secreted in minute amounts by various cells in response to viruses, double stranded RNA, mitogens, antigens and other diverse inducers. They possess both antiviral and antiproliferative activities in vitro and in vivo. There is a high degree of species specificity in the biological action of these materials with resultant emphasis on the human system. Human interferons have been divided into 3 classes based on their antigenicity and their physical and chemical properties: leukocyte( $\alpha$ -)interferon; fibroblast( $\beta$ -)interferon and immune( $\gamma$ -)interferon. Two of these,  $\alpha$ - and  $\beta$ -interferon, have been cloned in *E. coli* as a prelude to producing large amounts of human recombinant interferon for use in clinical studies of the treatment of cancer. The third member,  $\gamma$ -interferon, has proved elusive owing to extremely small quantities of immune interferon messenger RNA in leukocytes.

Previously, we reported the isolation and characterization of  $\gamma$ -interferon messenger RNA from normal, mitogen-stimulated, human leukocytes using ribonucleoside-vanadyl complexes as nuclease inhibitors. Subsequently, a recombinant plasmid containing a cDNA sequence coding for human  $\gamma$ -interferon has been constructed and characterized.

In order to obtain interferon messenger RNA in sufficient quantity for cloning, leukocytes from individual donors were screened for their ability to produce  $\gamma$ -interferon when induced with staphylococcal enterotoxin B and desacetylthymosin  $\alpha_1$ . Messenger RNA from the cells of a single, highly productive individual was extracted by methods developed in this laboratory and used to prepare polyadenylated molecules. The mRNA was further fractionated electrophoretically in denaturing agarose gels. A fraction sedimenting at 18S that consistently gave rise to significant levels of active  $\gamma$ -interferon when translated in *Xenopus laevis* oocytes was recovered. Double stranded cDNA was synthesized with reverse transcriptase using the 18S mRNA fraction as a template. Molecules larger than 800 base pairs were selected, extended with "tails" of polydeoxycytidylate and annealed to deoxyguanylate-tailed *Pst* I cleaved pBR322. The recombinant plasmids were used to transform *E. coli* K-12 strain 294. A cDNA library containing 8300 bacterial clones was produced and grown in duplicate on nitrocellulose for subsequent *in situ* screening procedures. DNA from the colonies of one set was hybridized with single stranded [ $^{32}$ P]cDNA reverse transcribed from 18S mRNA from induced cells. The second set of colonies was hybridized with [ $^{32}$ P]cDNA prepared from 18S mRNA extracted from uninduced (resting) leukocytes. The latter mRNA did not contain molecules coding for  $\gamma$ -interferon. Comparison of the hybridization intensities of both sets revealed that: (1) at least a third of the colonies hybridized to neither probe; (2) a few colonies hybridized better to the probe prepared from uninduced cells than that from induced cells and, most importantly, (3) 124 colonies hybridized specifically to the probe prepared from induced leukocytes. Plasmid DNA from the latter colonies was isolated, bound to nitrocellulose filters and rescreened with both probes. Only 22 colonies of the original 124 hybridized solely to [ $^{32}$ P]cDNA complementary to mRNA from stimulated cells. These colonies all represented induced sequences transcribed in leukocytes after treatment with a mitogen. The cDNA inserts from several of these clones were mapped with restriction endonucleases and, as a result of their apparent relatedness, a [ $^{32}$ P] DNA probe was prepared from one of them by nick translation and hybridized to the 22 candidates for  $\gamma$ -interferon as well as the entire clone bank. All 22 candidate clones hybridized and approximately 1 in 120 clones from

the original cDNA library also recognized the labeled cDNA. These results indicated that multiple copies of a single sequence derived from 18S mRNA from induced cells had been cloned. Whether this sequence coded for all or part of the  $\gamma$ -interferon molecule remained to be seen.

Using the clone with the largest insert, 1200 base pairs, the complete nucleic acid sequence was determined by the Maxam-Gilbert procedure and by the dideoxynucleotide chain termination method after subcloning in M13. The 3' end could be recognized by a "string" of consecutive adenylate residues. The 5' end was analyzed by comparing the sequence of the insert, itself, with that of cDNA molecules generated by reverse transcriptase using, as primer, a synthetic tridecanucleotide complementary to nucleotides 98-110 of the insert. The cDNA contained 15-20 nucleotides missing from the 5' end of the insert, hence the cloned insert specified almost the entire potential  $\gamma$ -interferon mRNA. The sequence of nucleic acids, however did not permit positive identification of the clone. The amino acid sequence of  $\gamma$ -interferon needed for comparison, was completely unknown.

Positive identification of the 1200 base pair insert was obtained in two ways. First, the insert, after suitable manipulation, was expressed both in *E. coli*, and in monkey cells using an SV-40 derived vector. In each case the antiviral activity present in the extracts behaved like authentic  $\gamma$ -interferon: it was pH 2 labile, sensitive to 0.1% sodium dodecylsulfate, refractory to neutralization by antibodies to either  $\alpha$ - or  $\beta$ -interferon and capable of being neutralized by an antibody made to partially purified  $\gamma$ -interferon. Second, messenger RNA which selectively hybridized to the insert was found to code for  $\gamma$ -interferon when translated in frog oocytes. Other clones, used as controls, did not bind molecules with  $\gamma$ -interferon mRNA activity.

Based on the evaluation of the nucleic acid sequence of  $\gamma$ -interferon, the protein consists of a polypeptide 166 amino acids long, 20 amino acids of which probably code for a signal sequence absent from the mature protein. The messenger RNA is considerably larger than that needed to encode a protein with a "subunit molecular weight" of approximately 17,000, excluding possible covalently bound carbohydrate. It contains approximately 600 nucleotides in the 3'-untranslated region. In the translated region there is a trivial amount of sequence homology with either  $\alpha$ - or  $\beta$ -interferon. Unlike the latter types of interferon, there is a single gene with introns for  $\gamma$ -interferon. Discrepancies between the apparent large size of the mRNA in denaturing gels and the smaller size of the full length clone, on the one hand, and the size of the protein specified by the mRNA and the much larger apparent size of the active protein have yet to be resolved.

Significance to Biomedical Research and the Program of the Institute: This project conforms to the Objective #3, Approach #1 of the National Cancer Plan. Its aims at an understanding of the biochemical control mechanisms by which normal cell growth and function are maintained. Disordered cell growth in neoplastic populations may then be better understood and rational attempts made to prevent or modify it.

The project also conforms to Objective #2, Approach #2. Since interferon produced *in vivo* or administered as an external agent may be effective in preventing or curing some types of cancer, it is essential to understand both

its modes of production and its mechanism of action in normal, virus-treated and malignant cells.

Proposed Course of Research: The gene expression of rare mRNAs, including  $\gamma$ -interferon, in resting and activated lymphocytes will be studied.

Publications:

Gray, P.W., Leung, D.W., Pennica, D., Yelverton, E., Najarian, R., Simonsen, C.C., Derynck, R., Sherwood, P.J., Wallace, D.M., Berger, S.L., Levinson, A.D. and Goeddel, D.V.: Expression of human immune interferon cDNA in *E. coli* and monkey cells. Nature 295: 503-508, 1982.

Puskas, R.S., Manley, N.R., Wallace, D.M. and Berger, S.L.: The effect of ribonucleoside vanadyl complexes on enzyme catalyzed reactions central to recombinant DNA technology. Biochemistry, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08215-05 LPP												
PERIOD COVERED October 1, 1981 to September 30, 1982														
TITLE OF PROJECT (80 characters or less)  Isolation and characterization of the angiogenesis factor(s)														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT														
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: K. Raju</td> <td style="width: 33%;">Staff Fellow</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Other: P.M. Gullino</td> <td>Chief, Lab. of Pathophysiology</td> <td>LPP, NCI</td> </tr> <tr> <td>J. Alessandri</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td>M. Ziche</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> </table>			PI: K. Raju	Staff Fellow	LPP, NCI	Other: P.M. Gullino	Chief, Lab. of Pathophysiology	LPP, NCI	J. Alessandri	Visiting Fellow	LPP, NCI	M. Ziche	Visiting Fellow	LPP, NCI
PI: K. Raju	Staff Fellow	LPP, NCI												
Other: P.M. Gullino	Chief, Lab. of Pathophysiology	LPP, NCI												
J. Alessandri	Visiting Fellow	LPP, NCI												
M. Ziche	Visiting Fellow	LPP, NCI												
COOPERATING UNITS (if any)  None														
LAB/BRANCH Laboratory of Pathophysiology														
SECTION Office of the Chief														
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205														
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords)														
<p>Since copper appears to be an indispensable component of the angiogenesis process, the ability to induce new formation of capillaries in the cornea was tested for ceruloplasmin, the copper carrier of serum, for fragments of the ceruloplasmin molecule with and without copper, for heparin and for glycyl-2-histidyl-2-lysine bound or not to copper ions. The results showed that the 3 different molecules were all able to induce <u>angiogenesis</u> providing that they bound copper. Fragments of the ceruloplasmin molecule also induced angiogenesis, but only when copper was bound to the peptides. Highest efficiency at lowest dose was obtained in the corneal test when copper was bound to the ceruloplasmin molecule or a fragment thereof. The data are interpreted to indicate that <u>copper ions</u> are involved in the cascade of events conductive to angiogenesis but the carrier molecule may be of quite a different nature.</p>														



Objective: To elucidate the mechanism of angiogenesis.

Project Description:

Methods: (1) Ceruloplasmin containing different amounts of copper ions was isolated by ion exchange chromatography. (2) Ceruloplasmin was cleaved by tryptic digestion and the tryptic fragments were separated by gel filtration techniques. (3) Copper was attached to heparin and to glycyl-L-histidyl-L-lysine. (4) Angiogenic capacity of each sample was tested with the corneal angiogenesis test.

Major Findings: (1) Angiogenic capacity is localized in ceruloplasmin fragments containing copper. (2) A fragment, with 11,000 MW appears to show the highest angiogenic capacity at the lowest dose and was found to contain 50% of the copper atoms of the ceruloplasmin molecule. (3) Copper attached to heparin or glycyl-L-histidyl-L-lysine transforms the molecule into an effective "angiogenesis factor." \_

Significance to Biomedical Research and the Program of the Institute: Isolation of a ceruloplasmin fragment able to induce angiogenesis in nanogram quantities may permit the development of a RIA to be applicable to biopsy tissue. Previous work showed that angiogenic capacity is acquired by cell populations at high risk of neoplastic transformation. A RIA able to demonstrate concentration of components of the angiogenesis process in a tissue may predict high risk of neoplastic transformation.

Proposed Course of Research: To complete the project.

Publications:

Ziche, M., Jones, J. and Gullino, P.M.: Role of prostaglandin E<sub>1</sub> and copper in angiogenesis. JNCI (In press).

Raju, K., Alessandri, G., Ziche, M. and Gullino, P.M.: Ceruloplasmin, copper ions, and angiogenesis. JNCI (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08217-05 LPP
PERIOD COVERED <b>October 1, 1981 to September 30, 1982</b>		
TITLE OF PROJECT (80 characters or less)  <b>Early changes in mRNA metabolism in regressing MTW9 tumors</b>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: P.K. Qasba Others: P. Chomczynski	Expert Visiting Scientist	LPP, NCI LPP, NCI
COOPERATING UNITS (if any)		
LAB/BRANCH <b>Laboratory of Pathophysiology</b>		
SECTION <b>Office of the Chief</b>		
INSTITUTE AND LOCATION <b>NCI, NIH, Bethesda, MD 20205</b>		
TOTAL MANYEARS: <b>2.0</b>	PROFESSIONAL: <b>2.0</b>	OTHER: <b>0</b>
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<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>Expression of the genes coding for milk proteins has been studied in the growing and regressing <u>MTW9 tumors</u>. At the onset of regression <u>milk protein mRNA</u> levels are reduced.</p>		

Project Description:

Objectives: Mammotrophic dependent MTW9 tumor provides a model system to study the influence of hormones on growth and regression of tumor tissue. To explore the system in our laboratory we are characterizing the expression of genes coding for  $\alpha$ -lactalbumin, whey phosphoprotein, k-protein, 42K casein, 25K casein and X-casein.

Methods Employed:

1) Estimation of specific mRNA levels is performed using DBM-cellulose paper method described in project no: Z01 CB 08218-07.

2) Synthesis of specific milk protein mRNAs in growing and regressing tumors is measured in isolated nuclei by in vitro RNA synthesis in the presence of [ $^{32}$ P $\alpha$ ] UTP. The labeled in vitro synthesized specific mRNA from nuclei is then quantitated by hybridization to the plasmid DNA.

Major Findings:

The results show that the growing MTW9 tumors transcribe the genes coding for  $\alpha$ -lactalbumin, k-protein, 42K casein and 25K casein. Transcripts corresponding to whey phosphoprotein and X-casein show variable results. In many tumors there are undetectable levels of Wp and X-casein MRNAs. However, in others there are low detectable levels of these mRNAs. In regressing MTW9 tumors, 6 hrs or earlier after the removal of mammotrophic hormonal stimulus, the levels of these mRNAs is reduced by 20 to 80% of that of growing tumor.

Isolated nuclei synthesized in vitro these milk protein specific mRNAs. The regressing tumor nuclei showed similar RNA synthetic ability. Preliminary results show that in the case of nuclei isolated from regressing tumor a lower UTP incorporation activity and lower levels of polyadenylation of synthesized RNA was observed.

Significance to Biomedical Research and the Program of the Institute:

Studies on the milk protein gene expression during growth and regression of hormone dependent MTW9 tumors will help us to understand the relationship between the expression of differentiated function, growth and malignant transformation.

Proposed Course of Research: The experiments will be further carried out:

1) To study the transcription of milk protein genes and their flanking sequences during growth and regression of these tumors.

Publications:

Results to be published.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER 201 CB 08218-07 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Hormonal influences on whey milk protein gene expression in rat mammary explants		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:  Others:	P.K. Qasba P. Chomczynski J. Kulski Y. Topper	Expert Visiting Scientist Visiting Fellow Chief, Intermediary Metabolism  LPP,NCI LPP,NCI NIAMD LBM, NIADDK
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4.8	PROFESSIONAL: 4.0	OTHER: 0.8
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>Method of quantitating milk protein mRNAs in mammary tissues and in cultured <u>mammary explants</u> was developed. This method has been used to evaluate the effect of <u>insulin</u>, <u>hydrocortisone</u> and <u>prolactin</u> on the production of <math>\alpha</math>-lactalbumin, whey phosphoprotein and k-protein. Stimulatory effect on the production of <math>\alpha</math>-lactalbumin and whey phosphoprotein mRNA can be seen in the presence of insulin and hydrocortisone alone. Although prolactin enhances this effect, the results indicate the hydrocortisone and insulin but not prolactin plays a key role in the regulation of <math>\alpha</math>-lactalbumin and whey phosphoprotein mRNA levels.</p>		

## Project Description:

Objectives: 1) Development of an accurate method for the estimation of specific mRNA levels in mammary tissue, 2) to evaluate the role of prolactin and hydrocortisone in the regulation of the production of mRNAs coding for  $\alpha$ -lactalbumin, whey phosphoprotein and k-protein.

Methods Employed: 1) Quantitation of mRNAs: The method is based on hybridization of the RNA linked to DBM-cellulose paper (filters) with a nick-translated [ $^{32}$ P] plasmid probe. RNA isolated from fresh tissue is bound to DBM-cellulose filters (usually 2 to 6  $\mu$ g of total RNA per 11 mm circles). The filters were prehybridized, then hybridized to [ $^{32}$ P] plasmids, extensively washed and counted. Standard curves were obtained by hybridization of known amounts of pure RNA coding one of the following proteins:  $\alpha$ -lactalbumin, whey phosphoprotein, k-protein, 42K casein, 25K casein and X-casein. The constructed standard curve showed that within the estimated range of 100 pg to 1.5 ng mRNA there is a linear relationship between the amounts of hybridizing [ $^{32}$ P] probe and mRNA.

2) Agarose gel electrophoresis of RNA: Estimation of the presence of mRNA in total RNA extracted from tissues or cultured explants were also performed after agarose gel electrophoresis of the total RNA. RNA from the gels was transferred to DBM-cellulose paper and hybridized with nick-translated [ $^{32}$ P] plasmids. The cellulose paper was washed and then exposed to an x-ray film.

3) Explant cultures: Explants taken from mid-pregnant rat mammary glands about 1 mg each) were cultured in medium 199 up to 9 days in the presence or absence of insulin, hydrocortisone and prolactin. Concentration of hormones was 1  $\mu$ g/ml of medium.

Major Findings:

In our earlier studies we constructed and isolated pBR 322 plasmids carrying 6 milk protein mRNAs. This created a new possibility to study the mRNA metabolism in mammary tissue. We have developed method for quantitation of these milk protein mRNAs in cultured mammary explants.

$\alpha$ -Lactalbumin mRNA: The presence of low levels of  $\alpha$ -LA mRNA was detected in the explants, cultured in the medium containing only insulin even on the 9th day of culture. Explant cultures incubated in the presence of insulin and hydrocortisone showed increased levels of  $\alpha$ -LA mRNA. A further increase in the levels of  $\alpha$ -LA mRNA was observed after supplementing the insulin hydrocortisone medium with prolactin. However, the stimulatory effect of prolactin could be observed only in the presence of hydrocortisone.

Whey Phosphoprotein (Wp): Similar to  $\alpha$ -LA, the effect of prolactin on Wp-mRNA can be attributed to the presence of hydrocortisone. This was clearly shown in the explants cultured for 6 days in the presence of insulin. On the 6th day of culture there was no detectable amount of Wp-mRNA in the explants. Addition of prolactin caused no induction of Wp-mRNA. Supplementing the culture system with hydrocortisone, sensitized the explants to prolactin. On the other hand explants cultured for 6 days in the presence of insulin and hydrocortisone contain significant amounts of Wp-mRNA. It was concluded that the stimulatory effect of prolactin on the production of  $\alpha$ -lactalbumin and whey phosphoprotein mRNA depends on the presence of hydrocortisone.



k-protein: Estimation of k-protein mRNA in explant cultures under various hormonal combinations showed that the production of the mRNA is dependent on the cooperative action of insulin and hydrocortisone and prolactin. No k-mRNA was found in the explants cultured in the presence of insulin and hydrocortisone.

Significance to Biomedical Research and the Program of the Institute: Studies on the milk protein gene expression during normal differentiation of mammary cells will help us to understand underlying mechanism of dedifferentiation of the mammary epithelial cells during malignant transformation.

Proposed Course of Research: The experiments will be further carried out by gene transfer techniques to study the influence of hydrocortisone on the gene expression of WP and  $\alpha$ -LA.

Publications: Results to be published.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  ZO1 CB 08219-05 LPP									
PERIOD COVERED October 1, 1981 to September 30, 1982											
TITLE OF PROJECT (80 characters or less) cDNA sequence of $\alpha$ -LA, whey phosphoprotein & k-protein: their genomic methylation											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT											
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: P.K. Qasba</td> <td style="width: 33%;">Expert</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Others: A. Dandekar</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td>E. Appella</td> <td></td> <td>LPP, NCI</td> </tr> </table>			PI: P.K. Qasba	Expert	LPP, NCI	Others: A. Dandekar	Visiting Fellow	LPP, NCI	E. Appella		LPP, NCI
PI: P.K. Qasba	Expert	LPP, NCI									
Others: A. Dandekar	Visiting Fellow	LPP, NCI									
E. Appella		LPP, NCI									
COOPERATING UNITS (if any)											
LAB/BRANCH Laboratory of Pathophysiology											
SECTION Office of the Chief											
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0									
CHECK APPROPRIATE BOX(ES)											
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER											
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords) The sequence analysis of cDNA clones have shown: a) Rat $\alpha$ -LA is larger than any known $\alpha$ -lactalbumins. It has 17 extra residues beyond the COOH terminus of the $\alpha$ -lactalbumin isolated and sequenced to date from other species. The predicted COOH-terminal sequence is hydrophobic and proline rich and bears some resemblance to $\beta$ -casein sequences; b) <u>Rat whey phosphoproteins</u> contain high content of cysteine, glutamic acid, aspartic acid and serine, but lack tyrosine. The cysteines appear in unique arrangements and are repeated in two domains of the protein. The second domain has striking similarities with the second domain of the red sea turtle <u>protease inhibitor</u> ; c) <u>The encoded k-protein sequence</u> lacks in cysteine and has several potential phosphorylation sites. Methylation of $\alpha$ -LA, whey phosphoprotein and k-protein gene sequences in the rat mammary gland at various stages of functional differentiation and several rat mammary tumors have shown an inverse relationship between the expression of $\alpha$ -LA, Wp or k- gene and the <u>methylation</u> of this <u>gene sequences</u> .											

Project Description:

Objectives: The purpose of this work is: a) to obtain the information about the mRNA sequences for noncasein milk proteins--  $\alpha$ -LA, Wp and k-protein -- which is essential for understanding metabolism of these mRNAs; b) to obtain the aminoacid sequence of noncasein milk proteins from the corresponding cDNA sequences; c) relationship of DNA methylation with the expression of these genes.

Methods Employed:

1) NH<sub>2</sub>-terminal sequence analysis of in vivo and in vitro synthesized Wp-proteins. RNA complementary to Wp-cDNA clone was hybrid selected and translated in vitro in the presence of [<sup>35</sup>S]methionine or [<sup>3</sup>H]leucine. The radiolabeled protein or the protein isolated from milk was subjected to automated Edman degradation.

2) Pst I cDNA inserts were isolated from cDNA clones. The DNA restriction fragments labeled at 3' or 5' -ends were sequenced by the chemical method of Maxam and Gilbert.

3) For the studies on the methylation status of the gene sequences of  $\alpha$ -LA, Wp and k-proteins in mammary gland and mammary tumors, the DNA was digested with appropriate restriction enzymes and then subjected to Southern Blot analysis.

Major Findings:

1. Sequence analysis of cDNA clones:

a) The sequence analysis of rat  $\alpha$ -LA cDNA clones has shown that rat  $\alpha$ -LA is larger than any known  $\alpha$ -lactalbumins as was predicted from our previous protein data. It has 17 extra residues beyond the COOH terminus of the  $\alpha$ -lactalbumin isolated and sequenced to date from other species. The predicted COOH-terminal sequence is hydrophobic and proline rich and bears some resemblance to  $\beta$ -casein sequences. The results suggest an interesting possibility that all other  $\alpha$ -LAs are synthesized with the extensions at the COOH-terminal end as in the rat and during the secretory process, the COOH-terminal extension is cleaved off in a manner similar to a presequence.

b) The sequence analysis of whey phosphoprotein cDNA clones have shown that whey phosphoproteins contain high content of cysteine, glutamic acid, aspartic acid, and serine, but lacks tyrosine. The cysteines appear in unique arrangements and are repeated in two domains of the protein. The second domain has striking similarities with the second domain of the red sea turtle protease inhibitor. The whey phosphoprotein mRNA is detected during mid pregnancy in lactation in the rat mammary gland but is barely detected in mammary tumors in which other milk protein mRNAs are expressed.

c) The k-protein mRNA sequence encodes a protein which lacks cysteine. The encoded protein shows a homology to a part of SV40 T-antigen. The mRNA sequence also shows a region of homology with the DNA sequence found near the origin of replication of SV40 and Polyoma DNA.

2. Methylation status of  $\alpha$ -LA, Wp and k-gene sequences in rat mammary gland and in certain tumors:

Msp and Hpa II restriction endonucleases which recognize the sequence C-C-G-G (Msp I cleaves both 5'- $\text{C-C-G-G-3'}$  and 5'- $\text{C-C-G-G-3'}$  and sequences whereas Hpa II cleaves the sequences 5'- $\text{C-C-G-G-3'}$  and 5'- $\text{C-C-G-G-3'}$  but not the sequence 5'- $\text{C-C-G-G-3'}$ ) were used to study the methylation state of these sequences in  $\alpha$ -LA, Wp and k-genes in the rat mammary gland at various stages of functional differentiation and in several rat mammary tumors. It was found that: a) In the organs other than the mammary gland, C-C-G-G sites around these genes are highly methylated gene sequences of  $\alpha$ -LA, Wp and k-proteins. However, there is a steady increase in the proportion of these unmethylated sequences in the mammary gland during gestation, initiating around 8 to 10 day of pregnancy. This increase in the proportion of under-methylated gene sequences follows closely i) the increase in the mRNA sequences corresponding to these genes and ii) increase in the epithelial cell population known to occur in the mammary gland. During lactation these gene sequences are completely demethylated which parallel with the maximum expression of these genes in the gland; b) In MTW9 and MCCLX mammary tumors which synthesize  $\alpha$ -LA protein, the  $\alpha$ -LA gene sequences are demethylated. Whey phosphoprotein gene in these tumors is hypermethylated, correlating with the reduced expression of this gene; c) MNU and 7-12-DMBA tumors show an altered methylation profile for these gene sequences which does not resemble to any stage of the mammary gland development. This pattern is also different from the patterns obtained from kidney and liver DNA.

Significance to Biomedical Research and the Program of the Institute:

Studies on the milk protein gene expression during normal differentiation of mammary cells will help us to understand underlying mechanism of dedifferentiation of the mammary epithelial cells during malignant transformation.

Proposed Course of Research: The experiments will be further carried out:

1) To identify the cell types present in the mammary gland which have these demethylated gene sequences and 2) to determine if hormones are involved in demethylation of these gene sequences.

Publications:

Qasba, P.I., Dandekar, A.M., Sobiech, K.A., Nakhasi, H.L., Devinoy, E., Horn, T., Losonczy, I. & Siegel, M.: Milk protein gene expression in the rat mammary gland (1982). In Critical Reviews of Food Sciences & Nutrition (CRC press) 16(II), pp. 164-189.

Dandekar, A.M. & Qasba, P.K.: Rat  $\alpha$ -lactalbumin has 17-residue-long COOH-terminal hydrophobic extension as judged by sequence analysis of the cDNA clones Proc. Natl. Acad. Sci. USA 78: 4853-4857, 1981.

Dandekar, A.M. & Qasba, P.I.: Inverse relationship between  $\alpha$ -LA gene expression and methylation of the gene sequences (1980) Eur. J. Cell. Biol. 22: 10.

Dandekar, A.M., Robinson, E.A., Appella, E. and Qasba, P.K.: Complete sequence analysis of cDNA clones encoding rat whey phosphoprotein: Homology to a protease inhibitor. Proc. Natl. Acad. Sci. USA, July issue, in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  201 CB 08220-01 LPP						
PERIOD COVERED October 1, 1981 to September 30, 1982								
TITLE OF PROJECT (80 characters or less)  Structural gene organization of rat $\alpha$ -LA and its genomic DNA sequence								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 40%;">PI: P.K. Qasba</td> <td style="width: 30%;">Expert</td> <td style="width: 30%;">LPP, NCI</td> </tr> <tr> <td>Others: S. Safaya</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> </table>			PI: P.K. Qasba	Expert	LPP, NCI	Others: S. Safaya	Visiting Fellow	LPP, NCI
PI: P.K. Qasba	Expert	LPP, NCI						
Others: S. Safaya	Visiting Fellow	LPP, NCI						
COOPERATING UNITS (if any)								
LAB/BRANCH Laboratory of Pathophysiology								
SECTION Office of the Chief								
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205								
TOTAL MANYEARS: 2.0	PROFESSIONAL: 2.0	OTHER: 0						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords)  Rat $\alpha$ -lactalbumin genomic clones have been isolated from a rat liver DNA library. Arrangement of coding and intervening sequences have been established. This cellular gene is about 4 kb long, about 5 times the length of the structural gene.								

Project Description:

Objectives: The purpose of this work is: a) to establish the genomic organization of  $\alpha$ -lactalbumin gene and b) to determine the DNA sequence of the coding regions, of the junctions of the intervening sequences and of 5'-end flanking regions.

Methods Employed:

1) The amplified rat liver DNA library (in  $\lambda$  Charon 4A phage DNA) was screened using the in situ plaque hybridization techniques of Benton and Davis (Science 1977, 196, 180-187). Plaques containing  $\alpha$ -lactalbumin structural gene sequences were purified as described by Maniatis et al. (Cell 1978, 15, 687-701).

2) For restriction enzyme mapping DNA is digested and fragments separated on agarose gels. After visualization of the DNA bands by ethidium bromide staining, DNA is analyzed by Southern blots. Nick-translated [ $^{32}$ P] labeled p- $\alpha$ -LA 18 is used as a hybridization probe to localize the structural gene sequences for  $\alpha$ -LA. 5'-end labeled  $\alpha$ -LA mRNA is used as a hybridization probe to localize the 5'-end sequences.

3) DNA sequence analysis is carried out as follows: DNA restriction fragments are sequenced a) after labeling at 3' or 5'-ends by the chemical method of Maxam and Gilbert or b) by M13mp7 dideoxy sequencing technique of Messing et al. (Nucleic Acid Res., 1981, 9, 309-321).

Major Findings:

1)  $\alpha$ -Lactalbumin mRNA sequence of about 850 bases spans on a cellular gene which is about 4 kb long.

2) An intervening sequence has been identified in the genomic clone at a position corresponding to an amino acid residue 103 of rat  $\alpha$ -LA. The DNA sequence beyond the junction towards the 3'-end of the mRNA has been aligned with the cDNA sequence of the  $\alpha$ -LA cDNA clone.

The sequencing of the remaining part of the genomic clone is under investigation.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of the organization of the milk protein gene sequences and the 5'-end flanking sequences in normal and neoplastic mammary cells will help in understanding the altered gene expression in the transformed mammary cells.

Proposed Course of Research: The experiments will be further carried out to complete the work stated in the objectives.

Publications:

Results to be published.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08225-07 LPP
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PERIOD COVERED  
October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)  
Membrane structure and dynamics of normal and neoplastic mammary gland

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER  
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Pinto da Silva	Chief, Membrane Biology Section	LPP, NCI
Others:	C. Parkison	Chemist	LPP, NCI
	A. P. Aguas	Visiting Fellow	LPP, NCI

COOPERATING UNITS (if any)

Dr. A. Peixoto de Menezes, Institute of Pathological Anatomy, School of  
Medicine, University of Lisbon, Lisbon, Portugal

LAB/BRANCH  
Laboratory of Pathophysiology

SECTION  
Membrane Biology Section

INSTITUTE AND LOCATION  
NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER: 0.1
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS      ☐ (b) HUMAN TISSUES      ☐ (c) NEITHER

☐ (a1) MINORS    ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The topochemistry of membrane-associated glycoproteins and glycolipids was studied in ovary-dependent mammary carcinomas. Preliminary results revealed different partition of lectin receptors with plasma membrane halves in growing and regressing tumors. The topology of the same membrane components of normal (under different functional states) and preneoplastic cells is currently under investigation. Further experimental work using antibodies directed to hormone receptors and cancer antigens is planned.

## Project Description:

Methods: Normal (pregnant and lactating animals), neoplastic (DMBA and MTW9 carcinomas), and preneoplastic mammary gland tissues of the rat are fixed in glutaraldehyde and processed for "fracture-label." Lectin-colloidal gold conjugates (L-CG) are used to localize in situ specific glycolipids and glycoproteins in plasma membrane halves. The partition of these membrane components is analyzed by electron microscopy and compared after quantification of the density of the marker on both membrane halves.

Major Findings: In growing tumors, heavy density of Con A and WGA receptors is seen on exoplasmic membrane halves of freeze-fractured cells whereas protoplasmic membrane halves are virtually devoided of labeling. In regressing tumors there is preferential partition of the same receptors with exoplasmic membrane halves, a significant density of labeling being identified in protoplasmic membrane halves.

Significance to Biomedical Research and the Program of the Institute: A variety of cell surface changes have been correlated with transformation and tumor function; little is known, however, about tumor cell surface characteristics that are directly involved in malignancy and metastatic spread. It has been demonstrated that transformation may result in burial of membrane sites, not accessible to immunorecognition in intact cells. Those cryptic sites, presumed to be involved in homo and heterotypic adhesion of malignant cells, can be detected after freeze-dissection and reorganization of membranes as obtained by the "fracture-label" method. Here, we apply for the first time the technology recently developed in our laboratory to the analysis of membrane architecture in cancer cells vs normal cells.

Proposed Course of Research: Our main goal in this project is to characterize structural changes of the cell surface of malignant cells that are related to tumour invasion into surrounding normal tissues and formation of metastatic growths at distant sites. We plan to do experimental work in the two following areas: 1) Use of monoclonal antibodies and immunogold techniques to localize in situ prolactin receptors, casin, and tumor antigens in freeze-fractured membranes of normal, preneoplastic, and neoplastic epithelial cells of the mammary gland in vivo; 2) Definition of the topology of specific glycoproteins, glycolipids, and tumor antigens in tumor cell lines of varying malignant potential.

## Publications:

Peixoto de Menezes, A. and Pinto da Silva, P.: Dynamic morphology of the apical membrane of lactating cells viewed by freeze-fracture. In D.J. Allen, P.M. Motta, L.J.A. Didio (Eds.): Three-dimensional microanatomy of cells and tissue surfaces. Elsevier, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08226-06 LPP									
PERIOD COVERED October 1, 1981 to September 30, 1982											
TITLE OF PROJECT (80 characters or less) Hormones and Growth Factors in Development of Mammary Glands & Tumorigenesis											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT											
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%;">PI: B.K. Vonderhaar</td> <td style="width: 25%;">Research Chemist</td> <td style="width: 25%;">LPP, NCI</td> </tr> <tr> <td>Others: M. Bhattacharjee</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td>A.E. Greco</td> <td>Chemist</td> <td>LPP, NCI</td> </tr> </table>			PI: B.K. Vonderhaar	Research Chemist	LPP, NCI	Others: M. Bhattacharjee	Visiting Fellow	LPP, NCI	A.E. Greco	Chemist	LPP, NCI
PI: B.K. Vonderhaar	Research Chemist	LPP, NCI									
Others: M. Bhattacharjee	Visiting Fellow	LPP, NCI									
A.E. Greco	Chemist	LPP, NCI									
COOPERATING UNITS (if any)											
LAB/BRANCH Laboratory of Pathophysiology											
SECTION Office of the Chief											
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 2.25	PROFESSIONAL: 1.5	OTHER: 0.75									
CHECK APPROPRIATE BOX(ES)											
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER											
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords)											
<p>             This project is designed to understand the role of hormones and growth factors in normal <u>mammary gland development</u> and <u>differentiation</u>. We wish to understand how <u>milk-protein production</u> is controlled by various hormones. Studies include: 1) purification of <u>α-lactalbumin</u> from mouse mammary glands and preparation and characterization of an antibody, 2) examination of the role of <u>thyroid hormones</u> and <u>adrenal steroids</u> in synthesis and secretion of milk proteins in organ culture, 3) examination of the role of <u>epidermal growth factor</u> and mammary gland-derived <u>growth factors</u> in lobulo-alveolar development of the immature mouse mammary gland.           </p>											



## Project Description:

Objectives: The purpose of these studies is to investigate the role of various hormones and growth factors in mammary gland development, differentiation and tumorigenesis. The roles of prolactin, thyroid hormones and adrenal steroids in milk protein synthesis and secretion is emphasized. The role of epidermal growth factor (EGF) in lobulo-alveolar development of immature mouse mammary glands in whole gland culture was examined. The purpose was to define the conditions involved in normal and hyperplastic development of the gland and production of mammary-derived growth factors.

Methods Employed: C3H/HeN and BalbC mice were used. Mild hypothyroidism was induced by addition of 0.1% thiouracil to the drinking water. Hyperthyroidism was induced by addition of  $T_4$  to the drinking water. Purification of  $\alpha$ -lactalbumin was accomplished by using standard chromatographic and separation techniques. Whole gland and organ culture was performed using chemically defined serum-free medium supplemented with various hormones, growth factors, and metabolic inhibitors. Production of  $\alpha$ -lactalbumin and casein was determined by radioprecipitation or radioimmunoassays using mono-specific antibodies prepared against the milk-proteins. Lobulo-alveolar development of mammary glands was assessed by hematoxylin staining of whole mounted glands. Estrogen/progesterone priming of 4 week old BalbC mice was performed by inserting a pellet (cholesterol: progesterone: estradiol 1001:1000:1) under the skin of the animals for the indicated period of time. Mammary-derived growth factor (MF) was isolated from mammary tissue by homogenization in PBS containing 2% Triton X-100 followed by centrifugation at 20,000 xg for 20 min. EGF levels and MF concentration in the tissue extracts were determined by standard RIA techniques and competition for  $^{125}I$ -EGF binding to hepatic microsomal membranes.

Major Findings: Mouse  $\alpha$ -lactalbumin was purified to homogeneity from mammary glands of lactating mice. In brief, this involved homogenization of the tissue and preparation of a post-mitochondrial supernatant. After removal of the lipid layer and sonication,  $(NH_4)_2SO_4$  precipitation was performed in the presence of  $Mn^{++}$ . The  $\alpha$ -lactalbumin was recovered in the 50-75% saturation pellet. The recovered proteins were then passed through a Bio-gel P-100 column. Active  $\alpha$ -lactalbumin was recovered by precipitation with  $(NH_4)_2SO_4$  at 75% saturation. The pellet was resuspended and after dialysis, the proteins run through a Bio-gel P-30 column followed by chromatography on DEAE-Sephacel. The active  $\alpha$ -lactalbumin was eluted with a gradient of 20mM-500mM Tris pH 7.8. This elution resulted in a single protein peak with two distinct peaks of enzyme activity. The two peaks were collected separately and their molecular weights determined with 12% SDS-PAGE. An estimated molecular weight of 14,600 was obtained for both  $\alpha$ -lactalbumins. 2-D gels run showed 2 spots with the same molecular weight but distinct pIs (6.2 and 5.8).

From these observations we have concluded that mouse  $\alpha$ -lactalbumin exists in 2 distinct charged forms. Both forms have equal activity in the lactose synthetase assay system. The charged forms probably are the result of different amounts or types of carbohydrate residues.

A mixture of the two forms of  $\alpha$ -lactalbumin was treated with glutaraldehyde and injected subcutaneously at multiple sites in rabbits to produce antibody. The antisera obtained was tested by the Ouchterlony double diffusion technique and gave

a double band with the purified antigen. No cross-reactivity was seen with mouse caseins or mouse serum proteins.

We used this antibody to establish a RIA for mouse  $\alpha$ -lac. This RIA can detect as little as 0.25ng of  $\alpha$ -lac. Using this antibody and one developed previously against mouse caseins, we examined hormonal regulation of milk protein production at various stages of development and in organ culture.

By RIA we demonstrated that  $\alpha$ -lac was absent from mammary fat pads cleared of epithelium and present in trace amounts in glands of adult virgin mice. By mid-pregnancy 0.28  $\mu$ g/mg protein were present. Lactating mammary glands contained 15  $\mu$ g/mg protein while mouse milk had 38.1  $\mu$ g/mg protein. We also examined the  $\alpha$ -lac content of involuted mammary glands from primiparous mice in different thyroid states. There was no detectable  $\alpha$ -lac in glands of hypothyroid mice, 0.119  $\mu$ g/mg in euthyroid and as much as 0.426  $\mu$ g/mg in hyperthyroid glands.

Mid-pregnancy mammary tissue was cultured in the presence of insulin (I), hydrocortisone (F) and prolactin (PRL) in the presence and absence of thyroid hormones ( $T_3$ ). After 48 hr in culture with IFPRL, an increased synthesis of both  $\alpha$ -lac and casein was observed.  $T_3$  had little or no effect on casein synthesis when cultured tissue was examined after 48 hr. A 2-3 fold increase in  $\alpha$ -lactalbumin was found in the tissue at this time. The media was examined for effects of the hormones on secretion of milk proteins. A low level of casein was found in media both in the presence and absence of  $T_3$ , but thyroid hormone did not enhance secretion of these peptides.  $\alpha$ -lactalbumin was found in the medium to a significant extent with  $T_3$  enhancing secretion 3-4 fold. Since  $\alpha$ -lac is a secretory milk protein important in production of the milk sugar lactose, we examined synthesis, secretion and activity of both components of the lactose synthetase system (galactosyl transferase and  $\alpha$ -lac) as well as the product lactose. Lactose synthetase activity and  $\alpha$ -lac itself were enhanced by the presence of  $T_3$  in the media. No such increase was observed for galactosyl transferase. Using  $^{14}C$ -glucose as a substrate for endogenous lactose synthetase in mid-pregnancy explants, we found a 2-fold increase in lactose content of the culture medium with addition of  $T_3$  to the cultures.

The products of synthesis of milk proteins in organ culture were then characterized by SDS-PAGE. The immunoprecipitated products in both tissue and media were analyzed on 12% SDS gels. No differences in casein patterns were seen in either tissue or media in the presence or absence of  $T_3$ . However, the  $\alpha$ -lactalbumin produced in tissue in the presence of IFPRL showed a single peak, while in the presence of  $T_3$  two distinct peaks (I and II) were seen. Only a single peak was secreted into the medium even in the presence of  $T_3$ .

Since others had reported that rat  $\alpha$ -lac antibodies can also precipitate another milk protein called Kappa (K), we examined the possibility that the two peaks found in tissue in the presence of  $T_3$  represented  $\alpha$ -lac and K. Since K does not contain cysteine, we labeled explants for 48 hr with  $^{35}S$ -cysteine in the presence and absence of  $T_3$ . Again, we formed two peaks in tissue cultured with IFPRL $T_3$  indicating that  $T_3$  enhances synthesis of a second form of  $\alpha$ -lac.

We then determined which of these two forms was the one secreted into the media. Using parallel cultures labeled with either  $^{14}\text{C}$  or  $^3\text{H}$ -amino acids we co-electrophoresed immunoprecipitated products found in tissue and media in IFPRL  $\pm$   $\text{T}_3$ . The peak designated I (slower moving on the gel) was the one synthesized and secreted in the presence of IFPRL and secreted in the presence of IFPRL $\text{T}_3$ .

Since  $\alpha$ -lac in several species is known to be a glycoprotein, we attempted to examine whether the differences in peaks I and II were due to differences in the glycosylation of a common peptide in the presence of  $\text{T}_3$ . Tunicamycin inhibits core oligosaccharide addition of N-linked carbohydrates. It also can inhibit protein synthesis. Tissue was cultured in the presence of IFPRL  $\pm$   $\text{T}_3$  with and without added tunicamycin. Cultures were labeled 48 hr with both  $^{35}\text{S}$ -methionine and  $^3\text{H}$ -galactose and mannose. Total protein synthesis in all cases was inhibited 30-35%. Carbohydrate addition to proteins was inhibited by 45% in IFPRL and by 65% in IFPRL $\text{T}_3$ . Synthesis and glycosylation of  $\alpha$ -lac reflected that seen for total proteins. Both peaks of  $\alpha$ -lac in tissue cultured in IFPRL $\text{T}_3$  were affected to the same extent.

We examined the effects of various concentrations of hydrocortisone (F) on synthesis and secretion of milk-proteins in organ culture. The optimal concentration for casein synthesis was  $10^{-6}$  M while  $\alpha$ -lac synthesis was optimal between  $10^{-8}$  and  $10^{-7}$  M. At higher concentrations of F,  $\alpha$ -lac synthesis was inhibited. The presence of  $\text{T}_3$  in the cultures did not alter the concentration curves for F. Secretion of newly synthesized caseins and  $\alpha$ -lac into the media was inhibited by all concentrations of F examined in the presence of IFPRL. Addition of  $\text{T}_3$  to the media did not overcome the inhibition of casein secretion but did overcome the inhibition of  $\alpha$ -lac secretion at all concentrations of F even as high as  $10^{-6}$  M.

Preliminary results indicate that this disparity in optimal concentration of F required for casein and  $\alpha$ -lac synthesis as well as the inhibition of  $\alpha$ -lac synthesis at concentrations above  $10^{-7}$  M is eliminated if the natural adrenal steroids corticosterone (C) and aldosterone (A) are used in the cultures in place of F.

This latter steroid is a necessary component of culture media used to assess lobulo-alveolar development in whole gland cultures of immature mouse mammary glands. This system was used to assess the impact of epidermal growth factor (EGF) and mammary-derived growth factors (MF) on lobulo-alveolar development of the glands in the presence of I, PRL, F and A.

4 week old Balb C mice were implanted with  $\text{E}_2$ /Prog pellets. At various times the #2 thoracic glands were removed and cultured in the presence of IFPRLA  $\pm$  EGF or MF. After as long as 9 days of priming with the pellet, no detectable differences in mammary gland morphology were detected between primed and unprimed glands. Unprimed glands and glands primed for 3 and 6 days were unable to develop after 11 days in culture with IFPRLA. Glands from animals primed for 9 days or longer had extensive lobulo-alveolar development after culturing with IFPRLA. However, when animals were primed for 6 days, the glands could develop if 60 ng/ml of EGF was added to the culture media. This requirement for EGF was equally met by



addition of a MF obtained from Dr. Jamie Zwiebel of LPP. However, the growth obtained with MF on occasion looked hyperplastic. This could reflect the origin of the MF which is an extract of NMU or DMBA-induced rat mammary tumors.

We next determined if the lack of requirement of 9 day primed tissue for EGF reflects endogenous EGF carried into the culture by the tissue. Extracts of mammary and submaxillary glands were examined for EGF by RIA at various times after implantation of the E<sub>2</sub>/Prog pellet. E<sub>2</sub>/Prog priming resulted in a significant and progressive increase in EGF content of the submaxillary gland. A 4-fold greater concentration in EGF in primed vs unprimed animals was observed as early as 3 days after pellet implantation. No EGF was detected in the mammary extracts at any time point.

We examined two possibilities: 1) the tissue is very sensitive to very low (not detectable by RIA) levels of EGF and 2) the mammary gland in primed animals elaborates a growth factor for which EGF substitutes in culture. The first possibility was examined in 2 ways: Animals were primed for 9 days using testosterone pellets to elevate the EGF levels maximally. Glands from these animals were unable to respond to IFPRLA in culture suggesting that elevated EGF alone was not sufficient to prime the animals. Glands from E<sub>2</sub>/Prog primed animals were tested for their ability to bind <sup>125</sup>I-EGF. Glands from 6 day primed animals bound 15-20 times more EGF/mg tissue than glands of unprimed mice. EGF binding was specific to epithelial rich regions of the gland and undetectable in fat pad free of epithelium.

Since MF substitutes for EGF in culture and also can bind to EGF receptors, the mammary extracts which were negative for EGF were examined for the presence of a MF which competes for EGF binding to liver microsomes. No such MF was detected in extracts of fat pad or unprimed tissue. However MF was detected in epithelial rich regions of mammary glands primed with E<sub>2</sub>/Prog for as little as 3 days and continued to increase up to 9 days (the latest time point examined).

Thus, we tentatively conclude that E<sub>2</sub>/Prog priming enhances synthesis of a MF and its receptors on mammary epithelial cells of immature female mice. Whether this is a direct effect or mediated through EGF is to be determined. This MF in the presence of I, PRL, F and A promotes lobulo-alveolar development of the immature gland in culture.

Significance to Biomedical Research and the Program of the Institute: Prolactin-thyroid interactions are important in growth and differentiation of mammary glands. Altered thyroid status may be implicated in risk of human breast cancer. It is not yet clear from the literature whether the effects of thyroid hormones are primary (i.e. directly on the mammary gland) or secondary (i.e. through alterations in other hormones). Therefore, all aspects of thyroid hormone control of growth and differentiation of the normal gland as well as development of mammary tumors must be understood. The development of hyperplastic alveolar nodules (HAN) in mouse mammary glands has been correlated with subsequent mammary tumor development. Thus, we wish to examine those growth factors (both from normal and tumor tissue) which lead to lobulo-alveolar development and HAN formation in mammary glands in culture.

Proposed Course of Research: We will continue to study the effect of thyroid and adrenal hormones on milk protein synthesis and secretion. The two peaks of  $\alpha$ -lac seen in tissue cultured in the presence of IFPRLT<sub>3</sub> will be characterized and the mRNA produced will be examined to determine if the  $\alpha$ -lac forms result from two distinct polypeptides or are modified forms of the same peptide. The nature of the MF involved in lobulo-alveolar development and HAN formation will be examined. MFs from normal (E<sub>2</sub> Prog primed) glands as well as mammary tumors will be characterized in collaboration with Drs. J. Zwiebel and W. R. Kidwell.

Publications:

Alabaster, O., Vonderhaar, B.K. and Shafie, S.M.: Metabolic modification by insulin enhances methotrexate cytotoxicity in MCF-7 human breast cancer cells. Europ. J. of Cancer and Clinical Oncology 17: 1223-1228, 1981.

Lewko, W.M., Liotta, L.A., Wicha, M.S., Vonderhaar, B.K. and Kidwell, W.R.: Sensitivity of N-nitroso-methylurea-induced rat mammary tumors to cis-hydroxyproline, an inhibitor of collagen production. Cancer Res. 41: 2855-2862, 1981.

Smith, G.H. and Vonderhaar, B.K.: Functional differentiation in mouse mammary gland epithelium is attained through DNA synthesis inconsequent of mitosis. Developmental Biol. 88: 167-179, 1981.

Kidwell, W.R., Knazek, R.A., Vonderhaar, B.K. and Losconczy, I.: Effects of unsaturated fatty acids on the development and proliferation of normal and neoplastic breast epithelium. In Arnott, M.S., Van Eys, J. and Wang, Y.-M. (Eds.) Molecular Interrelations of Nutrition and Cancer. Raven Press, New York, NY, 1982, pp. 219-236.

Vonderhaar, B.K. and Smith, G.H.: Dissociation of cytological and functional differentiation in virgin mouse mammary gland during DNA synthesis inhibition. J. Cell Science 53: 97-114, 1982.

Vonderhaar, B.K.: Effect of thyroid hormones on mammary tumor induction and growth. In B.S. Leung (Ed.): Hormonal Regulation of Experimental Mammary Tumors. Pergamon Press, Inc., in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08229-06 LPP
PERIOD COVERED  October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Role of Dietary Lipids in Mammary Cancer		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <div> PI: W. R. Kidwell Other: John Shaffer </div> <div> Chief, Cell Cycle Regulation Section Chemist </div> <div> LPP, NCI LPP, NCI </div> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH  <del>Laboratory of Pathophysiology</del> SECTION  Cell Cycle Regulation Branch INSTITUTE AND LOCATION  NIH, NCI, Bethesda, Maryland 20205		
TOTAL MAN-YEARS: 0.75	PROFESSIONAL: 0.25	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Epidemiological and experimental animal studies have strongly indicated that the risk of <u>breast cancer</u> development is influenced by the level of <u>lipids</u> in the diet. Current evidence suggests that this effect of fat is manifest directly at the breast locus. Experiments with intact animals and with cultured epithelium or breast explants, have led to the formulation of a model in which the prolactin stimulated breast epithelium recruits fatty acids needed for growth from proximal fat cells. Prolactin triggers the release of histamine either from <u>mast cells</u> in the mammary gland or from the epithelium itself. <u>Histamine</u> then activates the neighboring fat cell's lipases with the consequent release of fatty acids. The prolactin primed epithelium then selectively takes up the <u>unsaturated fatty acids</u> , part of which become localized in membrane phospholipids, favoring proliferation by as yet, undefined means. Elevations in dietary fat above the buffering capacity of the mammary adipocytes may thus directly supply the stimulating fatty acids to the mammary epithelium bypassing the required recruitment step, which may be rate limiting for proliferation.		

## Project Description:

**Methods Employed:** Mammary ducts and alveoli were isolated from human, mouse or rat tissues or tumors by collagenase digestion and selective membrane filtration procedures. Cell cultures were grown in serum-free, lipid defined medium to establish growth responses to fatty acids. The uptake or release of free fatty acids by tissues or cells in culture was determined by fractionation of the growth media lipid fraction on thin layer plates followed by derivitization and analysis by high performance liquid chromatography or gas liquid chromatography. Antioxidants were included in the extraction, fractionation and derivitization steps and the quantities of fatty acids found were adjusted for losses utilizing recoveries of internal standards.

**Major findings:** 1. Prolactin stimulates the release of free fatty acids from mammary adipocytes. As little as  $5 \times 10^{-10}$  M prolactin effects the release of free fatty acids from explants of normal mammary tissue. The released fatty acids are almost certainly derived from the adipocytes of the explant since prolactin stimulates uptake rather than release of fatty acids by purified mammary epithelium in culture. Since the prolactin receptors of the mammary gland are confined to the epithelium the prolactin effect must be indirectly manifest through the epithelium. 2. Histamine may be an intercellular signal which functionally couples adipocytes and epithelial elements of the gland. The ability of prolactin to stimulate free fatty acid release from explants of mammary tissue containing both epithelium and adipocytes is blocked by Benadryl, an antihistamine which acts through H1 receptors on fat cells. Furthermore, histamine at physiological concentrations ( $2 \times 10^{-8}$  M) is a very effective stimulator of free fatty acid release from mammary fat cells. 3. The histamine which triggers adipocytes to release fatty acids probably originates from mast cells in the mammary gland. Mast cells are found in abundance in the mammary gland and are especially abundant in hormone dependent mammary tumors. Some mast cells have been found in close association with the glandular epithelium, separated from it only by a basement membrane. Purified preparations of epithelium contain about 1 mast cell per 1000 epithelial cells. When the mast cells are removed by serial passaging of the epithelium on collagen gels, prolactin does not stimulate histamine production. If, however, the mast cells are present, prolactin stimulates histamine release in amounts which are capable of stimulating free fatty acid release from mammary fat cells.

Significance to Biomedical Research:

In attempts to understand the role of dietary lipids in the development of breast cancer we have begun to analyze the types, quantities and sources of fatty acids taken up by mammary epithelium. Our studies indicate a special role of unsaturated fatty acids in the physiology of the glandular epithelium. These fatty acids are apparently recruited from proximal mammary adipocytes which release their stores of fatty acids in response to a signal from hormonally stimulated epithelium. The fat cells of the gland provide an effective buffer which normally restricts the availability of unsaturated fatty acids to the

epithelium. Exceeding the buffering capacity such as may occur in individuals on high fat diets may sensitize mammary epithelium to basal levels of circulating hormones and thereby increase the proliferation rate of the epithelium and expand the population at risk to transformation.

#### Proposed Course of Research:

It has already been demonstrated that histamine levels in the mammary gland are directly correlated with the degree of mammary cell proliferation. Furthermore, it has been demonstrated that antihistamines block the proliferation of the mammary epithelium in experimental animals. We will attempt to determine whether the effects of Benadryl seen in vivo are the result of a direct action on the mammary gland. For this purpose we will assess the effects of Benadryl on the proliferation of the mammary epithelium in explant cultures in which both the epithelium and adipocytes are present as well as determining whether the antihistamine affects the proliferation of the isolated epithelium. Additionally we will examine the effects of antihistamines on the growth of hormone dependent and independent rodent mammary tumors.

#### Publications:

Kidwell, W.R., Knazek, R.A., Vonderhaar, B.K. and Losonczy, I. Effects of unsaturated fatty acids on the development and proliferation of normal and neoplastic breast epithelium. In Wang, Y., Arnott, M.S. and Van Eys, J. (Eds.): Molecular Interrelations of Nutrition and Cancer, 34th Annual Symposium on Fundamental Cancer Research. M.D. Anderson Hospital and Tumor Institute Monograph, p. 219-236, 1982.

Monaco, M., Kohn, P.H., Kidwell, W.R. and Lippman, M.E.: Vasopressin: Action on WRK-1 rat mammary tumor cells. J. Natl. Cancer Inst. 68: 267-270, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08230-06 LPP												
PERIOD COVERED October 1, 1981 to September 30, 1982														
TITLE OF PROJECT (80 characters or less)  Hormonal Control of Mammalian Follicular Maturation and Oogenesis														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT														
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: R.A. Knazek</td> <td style="width: 33%;">Senior Investigator</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Others: S.C. Liu</td> <td>Chemist</td> <td>LPP, NCI</td> </tr> <tr> <td>A. Rotondi</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td>J.R. Dave</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> </table>			PI: R.A. Knazek	Senior Investigator	LPP, NCI	Others: S.C. Liu	Chemist	LPP, NCI	A. Rotondi	Visiting Fellow	LPP, NCI	J.R. Dave	Visiting Fellow	LPP, NCI
PI: R.A. Knazek	Senior Investigator	LPP, NCI												
Others: S.C. Liu	Chemist	LPP, NCI												
A. Rotondi	Visiting Fellow	LPP, NCI												
J.R. Dave	Visiting Fellow	LPP, NCI												
COOPERATING UNITS (if any)														
LAB/BRANCH Laboratory of Pathophysiology														
SECTION Cell Cycle Regulation Section														
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: 1.75	PROFESSIONAL: 1.5	OTHER: .25												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords) FSH stimulated the synthesis of <u>prostaglandins</u> (PG) E and F <sub>2</sub> α by rat ovarian <u>granulosa cells</u> in a dose-related fashion that was augmented by exogenous hCG. PRL also exerted marked effects on PG synthesis in FSH- or FSH/hCG-treated animals. In physiologic amounts, PRL increased the rates of PGE and PGF <sub>2</sub> α synthesis 2 to 3-fold whereas injections of supraphysiologic amounts of PRL decreased these levels. The effectiveness of PRL over such a narrow concentration range is in agreement with <u>in vitro</u> studies on ovarian steroidogenesis. The role of PG in ovarian function is being studied via a newly devised assay for prostaglandin receptors which allows further study of the role of PG in ovarian steroidogenesis and ovulation. Suppression of endogenous PRL secretion by bromocryptine causes an increase in PG binding to ovarian membranes, an effect that is reversed by replacement of PRL in physiologic amounts. The observations suggest that prolactin exerts its effects through the PG cascade and explains, in part, the mechanism by which infertility occurs in <u>hyperprolactinemic women</u> .														



## Project Description:

Methods Employed: Female Sprague-Dawley rats are hypophysectomized (HIFR) at 21 days of age and implanted subcutaneously with a silastic-encased diethylstilbestrol (DES) pellet. The presence of high concentrations of this estrogenic substance in the absence of gonadotrophins stimulates the proliferation of granulosa cells. Dispersal of such ovaries can be achieved by forcing the tissues through a stainless steel wire mesh and resuspending the granulosa isolates in incubation buffer. Mature animals were also treated with gonadotrophins prior to treatment with bromoergocryptine and/or graded doses of PRL. Microsomal membranes were prepared from homogenates of ovaries from these latter animals and used for PG receptor studies.

An assay procedure for prostaglandin E and F has been devised consisting of organic solvent extraction, silicic acid column chromatography, and specific radioimmunoassay. Serum progesterone levels were determined by specific radioimmunoassay.

Membrane suspensions were incubated with graded concentrations of [ $^3$ H]PGF $_2\alpha$  with or without 1.6  $\mu$ M PGF $_2\alpha$  in 10 mM Tris, pH 7.5, for 2 hr at 22°C to provide binding data for Scatchard analyses. Values of specific binding were obtained by incubating membrane suspensions with 8.5 nM [ $^3$ H] PGF $_2\alpha$   $\pm$  1.6  $\mu$ M PGF $_2\alpha$  for 2 hr at 22°C. After incubation, the membrane-PG mixture was mixed for 5 minutes at 22°C with an equal volume of 2% charcoal-0.05% dextran suspended in 10 mM Tris, pH 7.5, to adsorb the unbound PG. A 250  $\mu$ l aliquot of this membrane-PG-charcoal mixture was then layered atop 150  $\mu$ l of a 2.2M sucrose, 10 mM Tris cushion, pH 7.5, that had been prepared previously in the microfuge tubes. The samples were then centrifuged for 3 minutes at room temperature and then cut at a point mid-way between the tops of the sucrose buffer and the charcoal pellet. The charcoal-containing lower half, to which the free prostaglandin had adsorbed, was discarded while the top portion of the tubes containing the membranes and their bound prostaglandins were counted in a scintillation counter.

Major Findings: Patients suffering from hyperprolactinemia are found to be anovulatory and/or amenorrheic. The reason for this association has been unknown. Studies by other investigators have shown that the absence of PGF $_2\alpha$  within the ovarian complex prevents release of the ovum in such a manner that would permit subsequent successful fertilization. In addition, Prostaglandin E has been shown to induce neovascularization when implanted in the cornea of the rabbit. Other studies have shown that follicular neovascularization follows the ovulatory surge of LH, a necessary event in forming the well-vascularized corpus luteum, and that women suffering from hyperprolactinemia have an inadequate luteal phase. These observations suggested to us that prolactin might be exerting its effects on the ovary through modification of the PG pathways and prompted the following studies.

Granulosa cells were obtained from hypophysectomized, immature, female rats that had been treated with diethylstilbestrol implants and various s.c. injections of oFSH, PMSC, hCG, oPRL. Cell suspensions were incubated at 37°C for 2 hrs  $\pm$  10 $^{-5}$  M indomethacin. Prostaglandins E and F $_2$  were extracted from the suspensions, partially purified by chromatography on silicic acid columns and then quantitated by specific RIA. The amounts of PGE and PGF $_2\alpha$



synthesized by the DES-treated controls were not significantly altered by treatments with a single injection of 5 I.U. hCG and/or 100  $\mu$ g oPRL every 4 hours for 2 days prior to sacrifice. Three injections of 100  $\mu$ g oFSH, however, markedly increased the rates of synthesis of PGE and PGF<sub>2</sub> $\alpha$ . These rates were further increased by 40-100% when a single injection of hCG was added to the FSH treatment. Addition of hourly injections of 50  $\mu$ g oPRL to either the FSH or FSH + hCG schedule, however, decreased the synthesis of PGE by 79-80% and PGF<sub>2</sub> $\alpha$  by 35-56% during the two day treatment period. Doses of oPRL ranging from 0-50  $\mu$ g were also injected hourly for 48 hours into PMSG + hCG treated animals. This revealed that PG syntheses in the 1.0  $\mu$ g oPRL group being stimulated to 240-290% that of animals receiving no oPRL. Progesterone levels rose from 0 to 1 ng/ml in the sera of PMSG + hCG-treated animals as the dose of PRL was raised from 0 to 50  $\mu$ g/hr. These data suggest that the euprolactinemic and hyperprolactinemic states modify ovarian steroidogenesis and the ovulatory process by either accentuating or suppressing the FSH-related stimulation of prostaglandin synthesis.

The mechanism by which prostaglandins exert their effects are unknown. However, for these locally active hormones to be effective, they must bind to a receptor site within the ovary. Attempts to study PG binding have resulted in a rapid and highly reproducible PG receptor assay. The method, rapid and reproducible, has yielded Scatchard curves compatible with either negative cooperativity or the presence of both high and low affinity binding sites having  $K_d$  = 5.8 and 77 nM, respectively. Under the conditions of assay, free PGF<sub>2</sub> $\alpha$  was bound irreversibly to the charcoal. There was no significant diffusion of either membrane-associated or charcoal-associated PGF<sub>2</sub> $\alpha$  into the sucrose layer for up to 20 minutes after centrifugation, thereby removing any urgency for manipulation of the assay tubes. The PG-receptor complex was shown to be stable for at least 30 minutes, PG not being stripped from the membrane receptor. These observations demonstrate that the speed and stability of the assay are such that large numbers of samples can be studied within a short period of time.

Additional investigations showed that binding to the membranes reached equilibrium within 90-140 minutes when incubated at 22°C and was partially reversible with excess PGF<sub>2</sub> $\alpha$  displacing ~ 70% of the previously bound PGF<sub>2</sub> $\alpha$  within a 3 hour period. PGF<sub>1</sub> $\alpha$  and PGE<sub>1</sub> also displaced PGF<sub>2</sub> $\alpha$  but to a lesser degree while PGA<sub>2</sub> did not displace PGF<sub>2</sub> $\alpha$  from its receptor. When unlabeled prostaglandins E<sub>1</sub>, A<sub>2</sub>, F<sub>1</sub> $\alpha$  or F<sub>2</sub> $\alpha$  were mixed with [<sup>3</sup>H] PGF<sub>2</sub> $\alpha$  at the beginning of the initial 90 minutes incubation at 22°C, similar results were observed. For PGF<sub>2</sub> $\alpha$  to bind to ovarian membranes, prior exposure to gonadotrophins in vivo was required, maximal values being obtained 3-5 days after the first injection of FSH. Concomittant daily injections of 1 mg bromocryptine caused a two-fold increase in specific binding of PGF<sub>2</sub> $\alpha$  after three days of treatment. Injection of 1, 10, 100, or 1000  $\mu$ g PRL every 4 hours for 2 days resulted in a suppression of PG binding when the 10, 100, and 1000  $\mu$ g schedules were used.

The above studies indicate that prolactin modifies both prostaglandin synthesis and binding in the PRL-responsive ovary. This may be the mechanisms through which prolactin acts and may also prove to be the physiologic mechanism by which PRL receptor modulation is achieved. Extension of these observations to other PRL-responsive tissues is currently under investigation.

Significance to Biomedical Research and the Program of the Institute:

Knowledge of the mechanism by which prolactin acts upon the ovary will enhance our understanding of the role of this mammotrophic hormone in the development of both mammary carcinomata and the pathologic states which predispose individuals to its occurrence.

Proposed Course of Research: Granulosa cells subjected to various hormone manipulations will be studied in detail with special attention to alterations in the enzymes and intermediates within the prostaglandin cascade and the modulation of prostaglandin receptor activity.

Publications:

Knazek, R.A., Christy, R.J., Watson, K.C., Lim, M.F., Van Gorder, P.N., Dave, J.R., Richardson, L.L., and Liu, S.C.: Prolactin modifies FSH-induced prostaglandin synthesis by the rat granulosa cells. Endocrinology 109: 1566-72, 1981.

Rotondi, A. and Knazek, R.A.: A rapid method for the measurement of prostaglandin receptors. Prostaglandins and Medicine. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08235-05 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Membranes of human normal, thrombasthenic and Bernard-Soulier platelets		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: P. Pinto da Silva J. Chevalier	Chief, Membrane Biology Sec. Fogarty Visiting Fellow	LPP, NCI LPP, NCI
COOPERATING UNITS (if any) Dr. G. A. Jamieson, Red Cross Research Laboratory, Bethesda; Dr. J.F. David-Ferreira, Laboratory of Cell Biology, Gulbenkian Institute of Science, Oeiras, Portugal		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Membrane Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.6	OTHER: 0.1
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
Using the recent technique of <u>fracture-labelling</u> (developed in our laboratory) in which cytochemical studies are performed on cells that have been previously freeze-fractured, we determined the <u>partition and the distribution of Wheat Germ Agglutinin (WGA) and Concanavalin A (Con A) receptors in freeze-fractured plasma membranes of platelets from normal donors and from patients with Glanzmann's thrombasthenia (GT) and Bernard-Soulier Syndrome (BSS).</u>		

Methods:

Human platelets were isolated, fixed, embedded into a cross-linked matrix of bovine serum albumin, impregnated in glycerol, frozen and freeze-fractured. After thawing, the fractured cells were treated with WGA and Con A and pre-labeled with colloidal gold. Samples were either processed for thin section or critical point dried and platinum-carbon replicated.

Major Findings:

Platelet aggregation and adhesion to the subendothelium are surface mediated phenomena that involve plasma membrane components. Some lectins are able to interact with these components to activate platelets, stimulating both the release of contents of intracellular storage organelles and the platelet aggregation. In normal as well as in GT and BSS platelets, WGA and Con A binding sites appear to be exclusively associated to membrane components - glycoproteins and/or glycolipids - that, upon fracture, partition with the outer membrane half. In Bernard-Soulier platelets, the major membrane sialoglycoprotein GP Ib, involved into the adhesion process to the subendothelium, is missing. However, large number of WGA binding moieties still remain accessible for labelling in BSS cells, since only a slight reduction of colloidal gold can be observed as compared to normal platelets. This finding is in contrast with the current assumption that WGA binding sites were almost exclusively associated to GP Ib and its labile part, Glycocalicin GP Is. In platelets from Glanzmann's thrombasthenia, despite the absence of GP IIB and GP IIIa major glycoproteins (involved in the aggregation process and strongly labeled with 125-I-labelled Con A), the Con A label did not differ from normal cells, suggesting a drastic modification of the membrane of GT cells as compared to normal platelets.

Significance to Biochemical Research and the Program of the Institute:

In recent years, the application of highly sensitive biochemical techniques have lead to detailed descriptions of the composition of the platelet membranes. However, little is known on the molecular organization of these components within the plane of the membrane. The ability of "fracture-label" techniques to visualize both the inner core of the membrane and the cytochemical markers allows definition of the nature and the spatial arrangement of the platelet membrane components. This technique gives new insights on the data obtained in biochemical studies, e.g. with SDS-PAGE electrophoretic techniques, enzymatic degradation.

Proposed Course of Research: Investigation of the patterns of partition and distribution of lectin receptors during platelet activation and aggregation.

Publications:

Chevalier, J. and Pinto da Silva, P. Fracture-label studies of the plasma membranes of human normal, thrombasthenic and Bernard-Soulier platelets. Blood (submitted).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08247-04 LPP								
PERIOD COVERED October 1, 1981 to September 30, 1982										
TITLE OF PROJECT (80 characters or less)  Basement Membrane Collagen Degradation and Its Role in Basement Membrane Physiology										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">L. Liotta</td> <td style="width: 35%;">Senior Investigator</td> <td style="width: 15%;">LPP, NCI</td> </tr> <tr> <td>Others:</td> <td>T. Kalebic</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> </table>			PI:	L. Liotta	Senior Investigator	LPP, NCI	Others:	T. Kalebic	Visiting Fellow	LPP, NCI
PI:	L. Liotta	Senior Investigator	LPP, NCI							
Others:	T. Kalebic	Visiting Fellow	LPP, NCI							
COOPERATING UNITS (if any) Laboratory of Developmental Biology and Anomalies, NIDR and Laboratory of Chemistry, NIAMDD										
LAB/BRANCH Laboratory of Pathophysiology										
SECTION Office of the Chief										
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205										
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5								
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS										
SUMMARY OF WORK (200 words or less - underline keywords)  There are at least five genetically distinct collagen types whose degradation may be controlled independently. The initial step of collagen degradation is performed by collagenase. We were the first to find that type IV basement membrane collagen and type V collagen is not degraded by human skin collagenase suggesting that a separate collagenase may degrade types IV and V collagen. <u>A collagenase which preferentially degrades type IV collagen</u> has been derived from metastatic tumor cells and from mammary epithelium. This collagenase has been purified 1000-fold and its cleavage products have been partially characterized. We are further studying the secretion rate of this enzyme by a wide variety of cell types both normal and malignant. <u>A collagenase which preferentially degrades type V collagen</u> has been identified in the conditional media.										



Project Description:

Objectives: The objectives of this project are to purify and characterize type IV collagen and type V collagen degrading enzymes and to study their biological significance in tumor invasion and metastases, angiogenesis, embryology and and diabetes.

Methods Employed: Crude collagenase is obtained from a) serum-free cultures of highly metastatic mouse tumor, b) serum free culture of minced involuting mammary gland, c) serum-free cultures of cultured human breast carcinoma and d) cultures of rat mammary ducts and alveoli. The enzyme activity is precipitated with ammonium sulfate and purified by HPLC molecular sieve and collagen affinity chromatography. Collagenase activity is studied on labeled and unlabeled purified collagens I, II, III, IV and V. Degradation products are studied by gel electrophoresis and electron microscopy. Enzyme specificity is studied by injecting the purified enzyme with or without inhibitors into various anatomical sites in the mouse.

Major Findings: A neutral protease has been extracted from the media of cultured metastatic tumor cells and purified approximately 1000 times after sequential ammonium sulfate fractionation, concanavalin A column chromatography, and molecular sieve chromatography. The protease has an apparent molecular weight of 70-80,000, is inactive at acid pH, requires trypsin activation, and is inhibited by ethylenediamine tetraacetic acid, but not phenylmethyl-sulfonyl fluoride, N-ethyl maleimide, or soybean trypsin inhibitor. The enzyme produces specific cleavage products for both chains of type IV collagen isolated without pepsinization and apparently cleaves at one point in a major pepsin extracted from placenta type IV collagen. A type V collagenase was purified 8000-fold and found to have a molecular weight of 50 KD.

A wide variety of human and rodent cells were studied for type IV and type V collagen degrading activity. Inflammatory cells endothelial cells, epithelial cells but not normal connective tissue cells exhibited enzyme activity. Metastatic tumor cells secreted enzyme activity in proportion to their ability to produce spontaneous metastases in vivo. Antibodies to type IV collagenase consistently inhibited metastases from i.v. injected tumor cells.

Latent collagenase was present in tumor interstitial fluid. collagenase activity was enhanced in MCF-7 breast carcinoma cultured with insulin.

Significance to Biomedical Research and the Program of the Institute: Breakdown of basement membranes occurs during the transition from in situ to invasive carcinoma and during penetration of vessel walls by metastasizing tumor cells. The basement membrane collagen degrading enzyme identified and purified in this project may play a role in the cancer invasion process. Detection of the enzyme may be a means of predicting the metastatic potential of a tumor. Furthermore, human breast carcinoma cells have been shown to secrete latent collagenase which degrades stroma type I collagen. Hence the specificity of different collagenase species elaborated by tumor cells may influence their pattern of invasion.

Proposed Course of Research: a) Purification of type IV collagen degrading enzyme to near homogeneity using HPLC and isoelectric focusing b) purifying antibodies against this enzyme. c) Use of the enzyme to elucidate the structure of type IV collagen. d) The effect of type IV protease on the morphologic structure of the basement will be studied by electron microscopy.

Publications:

Yaar, M., Foidart, J. M., Rennard, S., Brown, K., Martin, G. R. and Liotta, L. A.: Goodpasture-like syndrome induced by antibodies to basement membrane components. Am. J. Pathol. (in press).

Kidwell, W. R., Salomon, D. S., Liotta, L. A., Zwiebel, J. A. and Bano, M.: Growth factor effects on mammary epithelial cell proliferation and basement membrane synthesis. Cold Spring Harbor Proceedings 1981, (in press).

Salomon, D. S., Liotta, L. A., Rennard, S., Foidart, J. M. and Yaar, M.: Synthesis and turnover of basement membrane components by a teratocarcinoma cell OCT-10. Collagen and Related Diseases (in press).

Tryggvason, K., Liotta, L. A. and Salo, T.: Biosynthesis and turnover of basement membrane type IV collagen. In H. H., Scone (Ed.): New Trends in Basement Research (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08249-03 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Hormonal Control of Growth of Normal and Neoplastic Mammary Cells		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Mozeena Bano, Visiting Fellow, Cell Cycle Regulation LPP, NCI Others: W. R. Kidwell, Chief, Cell Cycle Regulation Section LPP, NCI Z. Zwiebel, Research Associate LPP, NCI D. Salomon, Expert LPP, NCI W. L. Lewko, Dept. Biochem. Univ. Louisville, Ky.		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Cell Cycle Regulation Section		
INSTITUTE AND LOCATION NCI, NIH Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.25	OTHER: 0.25
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS      X		
SUMMARY OF WORK (200 words or less - underline keywords) Production of <u>type IV collagen</u> is essential for the <u>growth</u> of normal mammary epithelium and <u>mammary adenocarcinomas</u> since selectively blocking the production of this protein causes growth arrest. Collagen production by adenocarcinomas is apparently constitutive because they produce factors that are potent stimulators of collagen synthesis. Mammary carcinomas which do not synthesize type IV collagen also do not produce the collagen synthesis stimulating activities. The <u>collagen synthesis stimulating activities</u> have been partially purified and shown to consist of two heat labile proteins with molecular weights of 68,000 and 6000 daltons. These factors differentially stimulate collagen synthesis in a variety of cell types but do not affect intra or extracellular collagen degrading activities. Since type IV collagen is synthesized by the <u>myoepithelial cells</u> of the adenocarcinomas, it is likely that the collagen synthesis stimulating factors act on the myoepithelial cells to increase their production of collagen or, they promote the conversion of stem cells in the tumor to the myoepithelial cell type.		

## Project Description:

Methods Employed: Primary mammary adenocarcinomas were induced with methylnitrosourea and the collagen synthesis stimulating activities extracted with acid-ethanol and further purified by gel filtration, ion exchange chromatography or preparative isoelectric focusing. The effects on collagen synthesis were assessed in various cell cultures either by determining the relative amount of labeled protein solubilized by purified collagenase or by quantitating the amount of 4-hydroxyproline and hydroxylysine produced per unit cell protein in the cell cultures.

Major Findings: 1. The major collagen synthesizing cells of the adenocarcinoma have been isolated. When the tumor cells are cultured in Calcium free medium, a spindle shaped cell, believed to be the myoepithelial cell, attaches to the culture dish but the epithelial cells do not. The two cell types have thus been isolated in essentially pure form. The basal cells synthesize type IV collagen and this protein represents 27% of the newly synthesized protein compared to only 0.4% of the protein made by the isolated epithelial cells. The latter cells contain estrogen receptor at 6 to 10 fold the amount of the myoepithelial cells. The myoepithelial cells synthesize a 56K form of keratin in amounts 10 fold more than do the epithelial cells. When co-cultivated, a large stimulation of cell growth is observed, compared to the growth of either cell type cultured separately. This indicates a positive interaction between the two cell types. 2. Adenocarcinomas produce two factors which differentially stimulate collagen synthesis. These factors apparently confer constitutivity in so far as collagen synthesis is concerned. The factors have molecular weights of 68K and 6K, are approximately neutral in charge and differentially stimulate collagen synthesis by 3-10 fold in NRK, 3T3, and normal mammary epithelial cells. Pulse-chase studies indicate that the factors act either by stimulating transcription and/or translation of collagen mRNA rather than affecting collagen turnover. The factors are about 4 fold more potent on a molar basis than EGF. The adenocarcinoma cells do not synthesize more collagen when purified tumor factors or EGF are added, indicating that the tumor factors are produced in sufficient amounts for maximal collagen synthesis. 3. Growth factors can be classified according to their ability to stimulate collagen production. A variety of hormones, growth factors, etc. have been screened for their ability to differentially increase collagen synthesis. In primary cultures of mammary epithelium the following are differential stimulators: hydrocortisone, EGF,  $\alpha_2$  macroglobulin, Embryonin, cholera toxin, the adenocarcinoma factors, and prostaglandin  $E_1$ . There are no differential effects on collagen synthesis by the following: Insulin, progesterone, estradiol, fibroblast growth factor, prolactin, platelet derived growth factor, thrombin, transglutaminase (factor 13), or prostaglandins  $F_2\alpha$  or  $E_2$ . Among those factors which differentially stimulate collagen synthesis, all except cholera toxin also stimulate cell growth.

Significance to biomedical research and the program of the institute: We have demonstrated that blocking collagen synthesis leads to the growth arrest of mammary adenocarcinomas. Further studies of collagen synthesis may provide a variety of methods by which tumor growth arrest can be accomplished in the breast cancer patient. These studies should provide fundamental information about the role of collagen in the growth and differentiation of normal breast epithelium.

Proposed course of research: The major objective is to further purify the collagen synthesis stimulating factors and determine whether their presence is indicative of hormonal responsiveness of mammary cancer. The studies will be extended from the rat model system to human tissues and the mechanism whereby the factors stimulate collagen synthesis will be further defined by use of in vitro translation systems and collagen C-DNA probes.

Publications:

Lewko, W.L., Liotta, L.A., Vonderhaar, B.K. and Kidwell, W.R.: Sensitivity of N-nitrosomethylurea-induced rat mammary tumors of cis-hydroxyproline, an inhibitor of collagen production. Cancer Res. 41: 2855-2862, 1981.

Kidwell, W.R., Liotta, L.A., Zwiebel, J.A., Salomon, D.S. and Bano, M.: Effects of growth factors on mammary epithelial cell growth and basement membrane synthesis. In Sato, G. and Sirbasku, D. (Eds.): Growth of cells in hormonally defined medium. Cold Spring Harbor Series on Growth Regulation, Vol. 9. In press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08250-02 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Fracture-label: Cytochemical labelling of freeze-fractured membranes		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: P. Pinto da Silva Chief, Membrane Biology LPP, NCI Others: M. Rosaria Torrisi Visiting Fellow LPP, NCI C. Parkison Chemist LPP, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Membrane Biology Section		
INSTITUTION NIH, Bethesda, MD 20205		
TOTAL YEARS: 0.6	PROFESSORIAL:	OTHER: .1
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> X <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Thin section and critical point dried fracture label (new cytochemical methods developed in our laboratory) are used to determine the partition and distribution of <u>glycophorin</u> associated wheat germ agglutinin (WGA) binding sites over fractured membrane halves of human erythrocytes. Most WGA is found on exoplasmic halves in contrast with preferential labelling of Band 3 over protoplasmic halves by concanavalin A. We conclude that fracture process of transmembrane proteins is stochastic in nature and appears modulated by the relative expression of each transmembrane protein at either surface as well as by their association to components of the erythrocyte membrane skeleton.		

#### Material and Methods:

Human erythrocytes ( $O^+$ ) are fixed in glutaraldehyde, embedded in 30% BSA, and gelled by glutaraldehyde. Gels are cut, impregnated in 30% glycerol and frozen. After fracture in liquid nitrogen the gel fragments are thawed, treated with WGA and labelled by colloidal gold coated with ovomucoid. They are then processed for thin section or critical point dried are replicated by platinum/carbon evaporation, and observed with an electron microscope.

#### Major Findings:

A detailed description of the ultrastructure of membrane halves from fracture-labelled preparations is provided and used to explain processes of supramolecular reorganization that occur upon thawing of freeze-fractured membranes. We show that during freeze-fracture only a minority of glycophorin molecules are dragged from the outer surface, across the exoplasmic half of the membrane and retained over the protoplasmic face. This contrasts with the opposite fracture behaviour of the other major transmembrane protein of erythrocyte membranes - Band 3 - which is preferentially labelled over the protoplasmic face, after being dragged across the outer membrane half. Analysis of the relative expression of these molecules at either membrane face, and of the extent of their association to components of the erythrocyte membrane skeleton lead us to conclude that the process of fracture of integral transmembrane proteins is of a stochastic nature and is modulated by several factors including the extent of the expression of amino acid chains and heterosaccharide at the outer surface and the existence and strength of molecular interactions with other membrane components at the inner surface. These findings not only complete our understanding of the process of fracture of human erythrocyte membranes, but they can be used in the interpretation of labelling patterns observed in other plasma and intracellular membranes (see in this annual report other projects of fracture-labelling).

#### Significance to Biomedical Research and the Program of the Institute:

Fracture-label techniques, developed in our section, appear to offer an enormous potential in the localization of membrane and cytoplasmic components. Their resolution is better than 20 nm and they have none of the limitations inherent to biomedical fractionation techniques (e.g. cross contamination, structural disruption). Every project started with this technique in our laboratory has had a success that far exceeds our expectations. Many laboratories are expressing interest in learning and attempting this new technology. We expect fracture-label to become a routine cytochemical and biochemical approach within the next few years.

**Proposed Course of Research:** Fracture-labelling methods are now being developed in a variety of other projects in my section. These involve substantial modification of the technique in order to adapt it to fracture of specialized cells (e.g. sperm) or of cells in monolayer culture. We intend also, if our personnel level is maintained, to pursue the development of autoradiographic techniques applied to the observation of the partition of lipids and proteins in freeze-fractured membranes.

Publications:

Pinto da Silva, P. and Torrisi, M.R. Freeze-fracture cytochemistry: Partition of glycophorin in freeze-fractured human erythrocyte membranes. Jour. Cell. Biol. 93: 463-469, 1982.

Pinto da Silva, P. and Torrisi, M.R. Partition of glycophorin and band 3 as observed in platinum replicas of critical point dried fracture labelled human erythrocytes. Jour. Cell. Biol. 91: 263a, 1981.

Pinto da Silva, P., Torrisi, M.R., Parkison, C., Kachar, B., Dwyer, N., Barbosa, M.L., Chevalier, J., Brown, C.: Fracture-label: Freeze-fracture cytochemistry of plasma and intracellular membranes. J. Cell. Biol. 91: 262a, 1981.

Pinto da Silva, P.: Fracture label cytochemistry: new approaches in labelling of plasma and intracellular membranes. Proceedings of the 10th International Congress of Electron Microscopy, Hamburg, August 1982 (invited lecture, in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08251-03 LPP																		
PERIOD COVERED October 1, 1981 to September 30, 1982																				
TITLE OF PROJECT (80 characters or less)  Growth Factor Production by Neoplastic Rat Mammary Epithelial Cells																				
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																				
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: J. Zweibel</td> <td style="width: 33%;">PHS Fellow</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>M. Bano</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td colspan="3"> </td> </tr> <tr> <td>Others: D. S. Salomon</td> <td>Expert</td> <td>LPP, NCI</td> </tr> <tr> <td>W. R. Kidwell</td> <td>Chief, Cell Cycle Regulation</td> <td>LPP, NCI</td> </tr> <tr> <td>P. M. Gullino</td> <td>Chief</td> <td>LPP, NCI</td> </tr> </table>			PI: J. Zweibel	PHS Fellow	LPP, NCI	M. Bano	Visiting Fellow	LPP, NCI				Others: D. S. Salomon	Expert	LPP, NCI	W. R. Kidwell	Chief, Cell Cycle Regulation	LPP, NCI	P. M. Gullino	Chief	LPP, NCI
PI: J. Zweibel	PHS Fellow	LPP, NCI																		
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COOPERATING UNITS (if any)																				
LAB/BRANCH Laboratory of Pathophysiology																				
SECTION Office of the Chief																				
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205																				
TOTAL MANYEARS: 2.8	PROFESSIONAL: 1.4	OTHER: 1.4																		
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES      X <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																				
SUMMARY OF WORK (200 words or less - underline keywords)																				
<p>             The program's objectives are to: 1) isolate and characterize a <u>mammary tumor factor(s) (MTF)</u> which resemble <u>epidermal growth factor (EGF)</u> and <u>sarcoma growth factor (SGF)</u> from chemically-induced <u>rat mammary tumors</u> and from the <u>conditioned medium</u> of primary cultures of mammary tumor epithelial cells; 2) to determine and compare the biological, biochemical and antigenic properties of MTF to EGF and SGF; 3) to compare the effects of MTF on the <u>growth and differentiation of normal rat mammary epithelium (RME)</u>, <u>mouse embryonal carcinoma (EC) cells</u>, and chick, mouse and rat fibroblasts and 4) to delineate the relationship of MTF production in <u>preneoplastic cells</u> and primary and transplantable <u>hormone-dependent and independent tumors</u> to tumor promotion and growth.           </p>																				

## Project Description:

Transformation of a variety of cells with RNA tumor viruses results in the failure of these cells to bind or respond to exogenous growth factors such as EGF or MSA because of the production of endogenous growth promoters such as sarcoma growth factor (SGF), which autostimulate these cells to continuously grow. If ectopic production of these or related growth factors is a general property of preneoplastic or neoplastic cells, then such factor(s) should be present in primary or transplantable, chemically-induced mammary tumors as well as in the conditioned medium of primary cultures of these tumor cells in vitro.

**Objectives:** These studies are designed to isolate and characterize ectopic growth factors from rat mammary tumors and from the conditioned medium of tumor cells in vitro and to ascertain if ectopic growth factor production is unique to mammary tumor cells or if normal RME cells also produce a similar or different set(s) of factors.

**Methods Employed:** A. Collection and Fractionization: Mammary tumors, mammary glands from perphenazine-treated rats, and other tissues will be extracted by an acid-ethanol procedure described by Roberts et al., PNAS, 77, 3495, 1980, for the isolation of transforming growth factors (TGF) from human carcinomas. The acid soluble material will be fractionated through a series of Amicon ultrafilters following dialysis against 1% acetic acid and further separation achieved by gel filtration, ion-exchange chromatography and preparative isoelectric focusing. Pooled material will be tested in several bioassay, radioreceptor and radioimmunoassay systems. Conditioned medium from primary cultures of tumor cells grown in serum-free medium will also be fractionated and tested in a similar manner. B. Bioassays: a) Ability to stimulate anchorage-independent growth (colony formation) of normal rat kidney (NRK) cells in soft agar; b) ability to enhance the proliferation of chick embryonic fibroblasts (CEF), mouse 3T3 fibroblasts, NRK and RME cells; c) ability to modulate EC differentiation with this parameter being monitored by the synthesis and turnover of type IV collagen and laminin. C. Biochemical assays: a) ability to compete with [ $^{125}$ I]EGF, [ $^{125}$ I]MSA or [ $^{125}$ I]insulin for specific binding to EGF, somatomedin or insulin receptors on EC cells or A431 human epidermoid carcinoma cells; b) ability to cross-react with either EGF or MSA in radioimmunoassays (RIA) using antisera to EGF, MSA or insulin and c) comparison of gel filtration, ion-exchange and electrophoretic properties to known growth factors.

**Major Findings:** The serum-free conditioned medium from primary cultures of 7,12-dimethylbenzanthracene (DMBA)-induced rat mammary adenocarcinoma cells contains a heat labile, acid-stable, protein factor(s) (mammary tumor factors, MTF's) which is able to compete with [ $^{125}$ I]EGF but not [ $^{125}$ I] insulin for radioreceptor binding on mouse EC cells. The factor(s) is antigenically distinct from EGF and induces the growth of NRK cells in soft agar as colonies. It is a mitogen for NRK, RME, CEF and 3T3 cells in monolayer culture, reduces the serum requirement for these cells in vitro and promotes the loss of contact inhibition of NRK cells. A similar activity (or set of activities) can be isolated directly from primary DMBA or nitrosomethylurea (NMU)-induced mammary tumors. Using several primary and transplantable mammary tumors, the factor(s) is highest in primary or transplantable tumors which are hormone-dependent. Activity is substantially lower in transplantable hormone-independent tumors tested (DMBA, NMU or MTW9A).



No activity can be detected in proliferating rat mammary glands. However, activity is present in tissue obtained from proliferating bovine mammary glands and in human breast epithelium obtained from reduction mammaplasty. The epithelium from primary DMBA-induced tumors has been partially fractionated into its two major cell components, epithelial cells and basal (myoepithelial) cells by selective attachment to culture dishes in the absence of calcium. The myoepithelial cell cultures (90-95% pure) and the epithelial cell fraction which is still contaminated with myoepithelial cells both produce the factor(s). The factor(s) are acid soluble, heat labile polypeptides having molecular weights of 6000 and 68,000. These two species can be further resolved by cation-exchange (CMC) chromatography. The larger form (68K) has a pI of approximately 5.2 while the smaller species (6K) is more neutral (pI, 7.6). Both the 68K and 6K species are able to induce NRK or 3T3 cell growth in soft agar as colonies and both selectively inhibit [<sup>125</sup>I]-EGF binding to its receptor on EC or A431 cells. A third biological activity has been detected and partially characterized in acid/ethanol extracts prepared from primary DMBA or NMU rat mammary adenocarcinomas. This activity differentially stimulates the synthesis of collagen in both RME cells (type IV collagen) and mesenchymal NRK cells (types I and III collagens). Whether this activity is physiochemically related to the soft agar colony stimulating activity and the EGF inhibitory activity is not known although partial resolution of this activity from the other two can be achieved by preparative isoelectric focusing. It has a molecular weight of approximately 68,000 and a pI of 5.9. Experiments are in progress to: (1) purify these factors from mammary tumors; (2) generate antibodies against these MTF's; (3) determine whether normal or malignant human mammary tissue possesses these MTF's; (4) correlate these activities with the endocrine status of the tumor (i.e. hormone dependent or independent); and (5) determine whether the production or activity of these MTF's can be modulated by hormones, growth factors or phorbol esters.

#### Significance to Biomedical Research and the Program of the Institute:

Transformation of cells in vitro by chemical or viral agents has been generally assumed to correlate with the acquisition of tumorigenicity in vivo. A variety of parameters are known to be associated with the transformed phenotype such as a loss in contact inhibition of growth; an increase in DNA synthesis, cellular proliferation and saturation density; ability to grow in soft agar and failure of cells which are able to differentiate to do so upon transformation. Several of these properties can be reversibly produced in nontransformed cells by factors which are synthesized by neoplastic cells in vitro. The ability to mimic the transformed phenotype by administration of these epigenetic, hormonal-like "transformation peptides" implies that they may be involved in the early stages of conversion of a normal cell to a preneoplastic cell. Autonomous production of such factor(s) may be involved in the acquisition of unrestrained growth by cancer cells. However, to date the data obtained in vitro with respect to the production, characterization and biological effects of these polypeptide factors such as sarcoma growth factor has not been extended in vivo in relationship to tumorigenicity. Moreover, although various sarcoma cells produce such factors, it is not known whether carcinoma cells produce similar or different set of agents. The experiments to be conducted should aid in resolving some of these questions.

Proposed Course of Research: To purify these factors from mammary tumors, to relate the production of these factors to the type of cell (preneoplastic or

neoplastic), and to determine whether these factors correlate with the endocrine status of the tumor (i.e. hormone dependent or independent).

Publications:

Zwiebel, J.A., Kohn, E., Davis, M.R., Salomon, D.S. and Kidwell, W.R.: Anchorage-independent growth-conferring activity from rat mammary adenocarcinomas. Cancer Res. (submitted for publication).

Kidwell, W.R., Salomon, D.S., Liotta, L.A., Zwiebel, J.A. and Bano, M. Growth factor effects on mammary epithelial cell proliferation and basement membrane synthesis. In Cold Spring Harbor Symposium on Cell Proliferation-Growth of Cells in Hormonally Defined Media, Vol. 9, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (in press).

Zwiebel, J.A., Kohn, E., Salomon, D.S. and Kidwell, W.R. Anchorage-independent growth conferring factor produced by 7, 12-DMBA induced rat mammary adenocarcinoma cells in culture. AACR Abstracts, Abst. No. 210, Washington, D.C. 1981, p. 53.

Bano, M., Zwiebel, J.A., Salomon, D.S. and Kidwell, W.R. Collagen synthesis stimulating activities produced by rat mammary adenocarcinomas. AACR Abstracts, Abst. No. 901, St. Louis, MO., 1982, p. 229.

Bano, M., Zwiebel, J.A., Salomon, D.S. and Kidwell, W.R. Production of collagen stimulating activity by rat mammary adenocarcinomas. J. Biol. Chem. (submitted for publication).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08252-03 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Interaction of Growth Factors & Tumor Promoters in Cell Growth & Differentiation		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: D. Salomon M. Paneerselvam  Others: A. Sahai L. Liotta	Expert Visiting Fellow Visiting Fellow Expert	LPP, NCI LPP, NCI LPP, NCI LPP, NCI
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4.0	PROFESSIONAL: 2.0	OTHER: 2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  The present studies are concerned with the role of <u>growth factors</u> such as <u>epidermal growth factor (EGF)</u> and those derived from rat mammary adenocarcinomas ( <u>mammary tumor factors</u> , MTF's) and their interactions with <u>phorbol ester tumor promoters</u> in relationship to <u>cell growth and differentiation</u> with respect to neoplastic transformation <u>in vitro</u> and <u>in vivo</u> . Several systems are being utilized to study the mechanism(s) by which EGF and MTF's function as modulatory agents for cellular growth and differentiation and the accentuation or attenuation of these responses by phorbol esters. A variety of biological endpoints are being examined including cell proliferation, <u>extracellular matrix (ECM)</u> production, phospholipid metabolism, protein kinase modulation and growth factor and <u>phorbol ester receptor</u> levels.		

## Project Description:

Tumor promoters such as phorbol esters function as co-carcinogens in vivo. In vitro phorbol esters are active in the nanomolar concentration range and reversibly confer upon cells several phenotypic traits which resemble those produced by chemical or viral transformation. The broad spectrum of biological effects produced by these compounds on different cell types at low concentrations are similar to the effects induced by various growth factors such as epidermal growth factor (EGF) and those polypeptides produced by transformed cells (transforming growth factors, TGF's). EGF has been shown to synergistically interact with phorbol esters in modulating cell growth. Specifically, EGF can act as a co-carcinogen in mouse skin, while phorbol esters are able to mimic the in vivo biological effect of EGF by causing precocious tooth eruption in neonatal rodents. Moreover, EGF and phorbol esters are able to cross-modulate receptors which mediate the response(s) to these two classes of agents.

Objectives: The effects of and mechanism(s) by which growth factors such as EGF and MTF's modulate in vitro cell growth and differentiation are being examined as these parameters might relate to tumor promotion and progression in vivo.

A. Effects of Growth Factors and Phorbol Esters on the Synthesis and/or Turnover of Components Associated with the Extracellular Matrix (ECM).

Major Findings: These studies are being conducted on human epidermoid carcinoma cells (A431) and on mouse embryonal carcinoma (EC) cells because both of these cell types synthesize and deposit a basement membrane (consisting of type IV collagen, laminin and fibronectin) which is required for their growth in vitro. Moreover, EC cells can differentiate in vitro into extraembryonic parietal endoderm (END) cells in a serum-free, hormone-defined medium. END cells are characterized by the appearance of plasminogen activator, enhanced synthesis of type IV collagen and laminin and the presence of a type IV-specific collagenase and a laminin degrading activity. Recent studies have demonstrated that (1) EGF can modulate the synthesis and/or turnover of type IV collagen in A431 and EC cells; (2) EC cells show a preferential attachment to and growth on type IV collagen-coated dishes, a response which is enhanced by laminin; (3) phorbol esters selectively and rapidly inhibit the binding of [ $I^{125}$ ]-EGF to cell surface receptors on EC cells; (4) this effect is mediated by the interaction of phorbol esters with specific, high affinity cell surface receptors which are distinct from the EGF receptors; and (5) EGF can modulate the down regulation of the phorbol ester receptor induced by phorbol esters. Experiments are in progress to determine the interactions between phorbol esters and growth factors (EGF, MTF's) on cell proliferation as these might relate to regulation of ECM production via changes in the receptors for these agents at the cell surface.

B. Effects of Growth Factors and Phorbol Esters on Phospholipid Metabolism and Cyclic-Nucleotide Independent Protein Kinases.

Major Findings: These studies are designed to delineate the mechanism(s) by which growth factors such as EGF and phorbol esters modulate cell growth and ECM production. Specifically, experiments are in progress to determine the sequence of biochemical events which are initiated following the binding of EGF and phorbol



esters to their respective receptors in human A431 and mouse EC cells. The A431 cells are being utilized because these cells are highly sensitive to EGF as they possess approximately  $1$  to  $2 \times 10^6$  receptor sites/cell. In contrast, EC cells

are being studied since EGF is a mitogen for these cells whereas it inhibits A431 cell growth. Moreover, although both cell types possess phorbol ester receptors, phorbol esters fail to inhibit EGF binding in A431 cells whereas they are extremely active as inhibitors of EGF binding in EC cells. Recent experiments have demonstrated that both EGF and phorbol esters rapidly stimulate (within 1 to 3 min.) the synthesis of phosphatidylinositol from [ $^3\text{H}$ ]-inositol with a subsequent generation of 1,2-diacylglycerol (1,2DG) and the release of arachidonic acid from the cells. These responses may be due to the activation of phospholipases  $\text{A}_2$  and C. Studies are in progress to determine: (1) whether there are soluble or particulate cyclic nucleotide-independent protein kinases in EC and A431 cells which can be rapidly activated following the application of EGF and/or tumor promoters to cells in vitro; (2) characterize those kinase(s) which are regulated by these agents to determine whether they are  $\text{Ca}^{++}$  dependent or independent kinases and whether they can be activated by phospholipids (PL or C kinase) such as 1,2DG or by polyamines (spermine and spermidine); and (3) identify and characterize the endogenous substrates for these kinases which might be potential mediators of EGF and phorbol ester effects on growth and ECM production.

#### Significance to Biomedical Research and the Program of the Institute:

Tumor promotion by compounds such as phorbol esters is a general phenomenon that has been observed in several tissues in vivo including skin, liver, mammary gland, lung, colon and bladder. Phorbol esters directly affect epithelial cells within these tissues by producing a set of reversible phenotypic changes that resemble the malignant or transformed state. Tumor promoters stimulate cell proliferation which in certain instances leads to an inhibition of cell differentiation. Cell proliferation and differentiation are normally controlled by specific hormones or growth factors. It has been proposed that phorbol esters may resemble growth factors or hormones with respect to the type of biological response(s) produced in certain cells to these compounds. Phorbol esters may be mimicking responses to an as of yet unidentified endogenous growth factor(s). Such a situation is not biologically unique since exogenous opiates utilize receptors for endogenous endorphins or enkephalins. The existence of such endogenous compound(s) would have obvious importance not only with respect to their normal function within the animal but also in relationship to tumor promotion and progression. The ongoing studies are utilizing several in vitro systems to delineate the interaction(s) of phorbol esters with growth factors such as EGF in a defined environment (serum-free medium). EC cells provide a system in which various aspects of cell growth and differentiation can be studied while A431 cells provide an appropriate model in which cell growth and modulation of certain differentiated parameters can be monitored. The ultimate goal is to utilize these systems to screen for potential endogenous "tumor promoters" which may be related to known or unknown growth factors or hormones.

#### Publications:

Salomon, D.S., Liotta, L.A. and Kidwell, W.R. Differential response to growth factors by rat mammary epithelium plated on different collagen substrata in serum-free, hormone-supplemented medium. Proc. Natl. Acad. Sci. USA 78 (1): 382-386, 1981.



Sando, J.J., Hilfiker, M.L., Salomon, D.S. and Farrar, J.J. Evidence that specific receptors mediate phorbol ester-enhanced production of T cell growth factor. Proc. Natl. Acad. Sci. USA 78 (2): 1189-1193, 1981.

Salomon, D.S. Inhibition of epidermal growth factor binding to mouse embryonal carcinoma cells by phorbol esters mediated by specific receptors for phorbol esters. J. Biol. Chem. 256: 7958-7966, 1981.

Sim, R.P., Salomon, D.S. Nylen, M.U. and Pratt, R.M. Tumor promoter TPA mimics epidermal growth factor-induced precocious tooth eruption in the rodent. Mutagenesis, Carcinogenesis and Teratogenesis 1: 361-365, 1981.

Salomon, D.S., Liotta, L.A., Rennard, S.I., Terranova, V., Foidart, J-M. and Yaar, M. Stimulation by retinoic acid of synthesis and turnover of basement membrane in mouse embryonal carcinoma-derived endoderm cells. Collagen and Related Res. 2: 93-110, 1982.

Salomon, D.S. and Smith, K.M. Attenuation by phorbol esters of epidermal growth factor-induced inhibition of proliferation of A431 epidermoid carcinoma cells, Abst. #204 Endocrine Society Ann. Meeting, San Francisco, CA, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08256-03 LPP									
PERIOD COVERED October 1, 1981 to September 30, 1982											
TITLE OF PROJECT (80 characters or less)  Role of Hormones and Cyclic Nucleotides in Mediating Cell Growth and Differentiation											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: W.B. Anderson</td> <td style="width: 33%;">Research Chemist</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Others: L. Nagarajan</td> <td>Visiting Scientist</td> <td>LPP, NCI</td> </tr> <tr> <td>C. J. Jaworski</td> <td>Chemist</td> <td>LPP, NCI</td> </tr> </table>			PI: W.B. Anderson	Research Chemist	LPP, NCI	Others: L. Nagarajan	Visiting Scientist	LPP, NCI	C. J. Jaworski	Chemist	LPP, NCI
PI: W.B. Anderson	Research Chemist	LPP, NCI									
Others: L. Nagarajan	Visiting Scientist	LPP, NCI									
C. J. Jaworski	Chemist	LPP, NCI									
COOPERATING UNITS (if any) Daniele Evain, Unite' INSERM 188, 74 Ave., Denfert, Rachereau 75014 Paris, France; S. Peter Nissley, Metabolism Branch, National Cancer Institute, NIH; Peter T. Mora, Macromolecular Biology Section, NCI, NIH											
LAB/BRANCH Laboratory of Pathophysiology											
SECTION Office of the Chief											
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.0	OTHER: 0									
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>											
SUMMARY OF WORK (200 words or less - underline keywords) Exposure of F9 teratocarcinoma cells to retinoic acid induces differentiation to a parietal endoderm cell type. <u>Cyclic AMP-dependent protein kinase</u> activity of the of the plasma membrane fraction is enhanced within 3 h of retinoid treatment of F9 cells and reaches a maximum at 15 h after the addition of retinoic acid. Retinoic acid treatment elicits a preferential increase in the amount of the R II regulatory subunit of the protein kinase associated with the membrane fraction. This suggests that the specific increase in type II cyclic AMP-dependent protein kinase associated with the plasma membrane may be an early event of retinoic acid action. The amount of a transformation sensitive 55 K protein decreases in F9 cells induced to differentiate with retinoic acid. Results suggest that a decrease in the amount of 55 K protein correlates with the onset of differentiation and not with <u>cell growth rate</u> . Low concentrations of <u>insulin</u> (20 to 40 ng/ml) stimulate the growth of F9 cells under <u>defined, serum-free conditions</u> . Insulin appears to stimulate the growth of these cells by acting directly through its own receptor.											

## Project Description:

**Objectives:** To investigate how adenylate cyclase activity, particularly its hormonal responsiveness, and cyclic AMP-dependent protein kinase activity is altered in malignant, undifferentiated cells to, in turn, influence cellular properties under cyclic nucleotide control such as the regulation of cell growth and differentiation.

**Methods Employed:** Cell culture, standard biochemical analysis of adenylate cyclase and protein kinase activities, hormone binding studies, SDS-polyacrylamide gel electrophoresis and radiographic analysis, immunoprecipitation and radio-immunoassay.

**Major Findings:** Embryonal carcinoma cells, the stem cells of teratocarcinomas, serve as a useful model for studying early events involved in embryonic differentiation and development. The acquisition of responsiveness to different hormones at various stages during embryonic differentiation probably plays an important role in mediating normal development. Treatment of F9 teratocarcinoma cells with retinoic acid results in growth arrest and differentiation into endodermal cells. Differentiation to the endoderm cell type markedly alters the adenylate cyclase response to calcitonin and parathyroid hormone; the cyclase of endodermal cells exhibits a low response to calcitonin while parathyroid hormone dramatically enhances cyclic AMP formation.

Preliminary studies have established that undifferentiated, pluripotent OTT 6050 stem cells exhibit very low fluoride- and hormone-stimulated adenylate cyclase activity. The ability of calcitonin to stimulate cyclic AMP production is dramatically increased prior to the onset of spontaneous differentiation to the endoderm cell type. Results suggest that the altered hormonal responsiveness is due to an alteration in the GTP coupling mechanism of the cyclase system.

Studies are in progress to determine possible ectopic hormone production by these early embryonic cell types, and to determine if hormone secretion is altered with cell differentiation. Initial results indicate that undifferentiated F9 cells secrete immunoreactive calcitonin, while parietal endoderm cells secrete immunoreactive parathyroid hormone. This lends support to the suggestion that ectopic production of, and altered hormonal responsiveness of adenylate cyclase and calcium transport systems to, these two hormones at specific stages in development may contribute to the regulation of subsequent steps of differentiation.

Retinoic acid treatment of F9 cells also has been shown to cause an increase in both cytosolic- and plasma membrane-associated cyclic AMP-dependent protein kinase activity. Kinase activity of the plasma membrane fraction is enhanced within 3 h of retinoid treatment and reaches a maximum at 15 h after the addition of retinoic acid. After 15 h of retinoid treatment there is only a 1.6-fold increase in the amount of the RI regulatory subunit of the protein kinase system associated with the membrane fraction, while membrane-associated RII regulatory subunit is increased 4.5-fold. These findings suggest that the increase in cyclic AMP-dependent protein kinase activity and the specific accumulation of RII associated with the plasma membrane fraction may be early events of retinoic acid action to mediate eventual cellular differentiation.

A specific cellular protein of Mr 55000 (55K) has been shown to be induced in several viral- and carcinogen- induced transformed cells. Results indicate that a similar protein is also present in OTT 6050 and F9 embryonal carcinoma cells. The amount of the protein decreases in F9 cells induced to differentiate to a parietal endoderm cell type with retinoic acid, as it does following spontaneous differentiation of OTT 6050 cells. The half-life of the 55 K protein was compared in the undifferentiated and differentiated cell types to determine if a change in stability might account, in part, for the altered levels of this protein. The 55 K protein is found to be most stable in SV40 - transformed cells and in undifferentiated F9 cells. It is significantly less stable in the more highly differentiated, untransformed cells. The results indicate that a decrease in the amount of 55 K protein correlates with the onset of differentiation of early embryonic cells, and not with cell growth rate. This protein appears to be expressed in undifferentiated embryonic stem cells. Certain transformations of other cell types apparently causes dedifferentiation to a stage where this protein once again is present at high levels. This protein may serve as a useful marker to follow embryonic cell differentiation and thus, to follow the transition from certain malignant to non-malignant cell types.

The appearance of specific receptors for growth-regulating substances during early stages of development could play a critical role in regulating specific growth during embryogenesis. Multiplication stimulating activity (MSA), an insulin-like growth factor, can replace the requirement for fetal calf serum for the growth of F9 cells under defined, serum-free conditions. Low concentrations of insulin (20 to 40 ng/ml) also stimulates the growth of F9 embryonal carcinoma cells under defined conditions. [<sup>125</sup>I] Insulin binding studies reveal the presence of high and low affinity receptor sites; insulin does not compete for [<sup>125</sup>I] MSA binding to F9 cells. The addition of antibodies to the insulin receptor (anti R) to serum-free growth medium also promotes F9 cell proliferation. Anti R blocks [<sup>125</sup>I] insulin binding to F9 cells, but does not alter the binding of [<sup>125</sup>I] MSA, indicating that anti R is exerting its growth-promoting effects by interacting with the insulin receptor. These results indicate that insulin is able to promote the growth of F9 cells by acting directly through its own receptor, and not through the MSA receptor present on F9 cells.

#### Significance to Biomedical Research and the Program of the Institute:

Embryonal carcinoma cells have the capacity, depending upon their environment, either to form tumors or to differentiate into normal cells. Thus, the study of teratocarcinoma cells allows the analysis of biochemical events relating to early mammalian development and to neoplasia. Treatments which result in the differentiation of stem cells within a tumor might negate the malignant cell type and result in a benign neoplasm.

Proposed Course of Research: Studies will continue on the elucidation of developmental aspects of the adenylate cyclase system, specifically concerning alterations in hormonal responsiveness. Other studies will attempt to establish if the ectopic production of calcitonin and/or parathyroid hormone contributes to the maintenance of the malignant state and to the regulation of subsequent steps of differentiation. This will involve studies on the mechanism of calcitonin action and its ability to alter calcium levels and thus activities under calcium



control. Since F9 cells secrete calcitonin attempts will be made to produce an inactive analog of calcitonin to antagonize the effect of this hormone.

To better understand how retinoic acid might act to cause the differentiation of F9 cells, studies are planned to elucidate the mechanism by which retinoic acid rapidly increases cyclic AMP-dependent protein kinase activity and selectively enhances RII interaction with the plasma membrane. Studies will continue on the role of somatomedins and insulin in regulating the growth of these early embryonic cells.

#### Publications:

Evain, D., Binet, E., and Anderson, W.B.: Alterations in calcitonin and parathyroid hormone responsiveness of adenylate cyclase in F9 embryonal carcinoma cells treated with retinoic acid and dibutyryl cyclic AMP. J. Cell Physiol. 109: 453-459 1981.

Pinkett, M.O., and Anderson, W.B.: Plasma membrane-associated component(s) that confer(s) cholera toxin sensitivity to adenylate cyclase. Biochim. Biophys. Acta 714: 337-343, 1982.

Plet, A., Evain, D. and Anderson, W.B.: Effect of retinoic acid treatment of F9 embryonal carcinoma cells on the activity and distribution of cyclic AMP-dependent protein kinase. J. Biol. Chem. 257: 889-893, 1982.

Nagarajan, L., Nissley, S.P., Rechler, M.M. and Anderson, W.B.: Multiplication stimulating activity stimulates the multiplication of F9 embryonal carcinoma cells. Endocrinology 110: 1231-1237, 1982.

Chandrasekaran, K., Mora, P.T., Nagarajan, L. and Anderson, W.B.: The amount of a specific cellular protein (p55) is a correlate of differentiation in embryonal carcinoma cells. J. Cell Physiol. (in press).

Nagarajan, L. and Anderson, W.B.: Insulin promotes the growth of F9 embryonal carcinoma cells apparently by acting through its own receptor. Biochem. Biophys. Res. Commun. (in press).



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08257-03 LPP
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Tumor cell invasion of native connective tissue (human amnion) in vitro.

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	L. A. Liotta	Senior Investigator	LPP, NCI
Others:	R. G. Russo	Visiting Fellow	LPP, NCI
	I. Margulies	Electron Microscopist	LP, NCI
	U. Thorgeirsson	Visiting Scientist	LP, NCI

COOPERATING UNITS (if any)

Laboratory of Pathology

LAB/BRANCH

Laboratory of Pathophysiology

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NIH, NCI, Bethesda, Maryland 20205

TOTAL MINORS:

PROFESSIONAL:

OTHER:

CHECK APPROPRIATE BOX(ES)

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1-0

☐ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Amnion invasion assay was used to quantitate migration of inflammatory and tumor cells through human connective tissue barriers. Polymorphonuclear leucocyte migration through the amnion was demonstrated by optical and electron microscopy. The rate of polymorphonuclear migration was stimulated by the chemoattractant N-formylmethionyl-leucyl phenylalanine (FMLP). Adherence studies of polymorphs to components of the basement membrane show that they use laminin to attach to type IV collagen. M5076 cells which secrete proteases to all the known types of collagen traverse the amnion within 24 hours. In the presence of the chemoattractant, FMLP, the rate of invasion is significantly increased. On the contrary, inhibition of invasion is seen with bovine cartilage extract and purified metalloproteinase inhibitor. Treatment, with crude and purified interferon of Ewing sarcoma cells increase their invasive capacity through the amnion basement membrane. Human or bovine endothelial cells grow to confluency on amnion basement membrane (BM). Tumor cell invasion is significantly retarded by the endothelial cell monolayer. Adult rat hepatocytes attach better to the amnion BM than to any of the five types of collagen tested and can be kept alive in a defined medium for three weeks.

## Project Description:

**Objectives:** 1) To study the basic mechanism of tumor cell invasion of basement membrane and factors which influence invasion. 2) To use the amnion basement membrane as a substrate for growing fastidious cells such as endothelial cells and normal hepatocytes.

**Methods Employed:** Normal full term placentas are used within 24 hours of delivery. The amnion is dissected from the chorion, the epithelial layer is removed and the membrane rendered nonviable with 0.1 M ammonium hydroxide the deepithelialized amnion washed thoroughly in PBS and stored in Eagles MEM with antibiotics. Untreated amnion is used for polymorph invasion. Amnion is placed in an invasion chamber with 8 micron Millipore filter in close contact with the stromal surface. Polymorphs or tumor cells are placed on the membrane and the number of cells which migrate through the whole thickness of the amnion and into the filter are stained and counted. Attachment studies involve counting stained cells on the amnion mounted on a microscopic slide. Treatment with various agents is performed either before or during the incubation in the invasion chamber.

**Major Findings:** 1) Polymorphonuclear migration through epithelium, human basement membrane and collagenous stroma is demonstrated in the amnion invasion system. A directed migration through the membrane is stimulated with a known leukocyte chemoattractant, FMLP. The response is dose dependent with the optimal FMLP concentration at  $10^{-8}$  M. Electromicroscopic examination of the polymorphs demonstrates attachment to the epithelium, movement between the epithelial cells, pseudopodia formation and focal dissolution of the basement membrane and finally migration through the collagenous stroma. 2) Polymorphs contain laminin on their cell surface and utilize it to attach to basement membranes. 3) Effects of a chemotactic agent (FMLP) and protease inhibitors on invasion of a highly invasive reticulum cell sarcoma line, M5076, are shown. FMLP stimulates invasion up to 6-fold without having any effect on type IV collagenase production. Crude bovine cartilage extract which contains mixture of protease inhibitors inhibits invasion 4 to 5-fold. A purified metalloprotease inhibitor isolated from rabbit bone culture medium inhibits invasion 4 to 5-fold. Soybean trypsin inhibitor, which is a serine protease inhibitor has no effect on invasion. All the agents tested are not cytotoxic and have no effect on cell attachment to the amnion. 4) Treatment of Ewing's sarcoma cells for 6 days with either crude or purified interferon (100 units/ml) leads to 2 to 4-fold increase in production of type IV collagenase by the Ewing's cells. The *in vitro* invasion studies of interferon treated Ewing's cells show up to 22-fold increase in invasion after 6 day incubation on the amnion. Similar results are seen when the Ewing's cells are treated with interferon during the 6 day incubation period on the amnion. 5) The amnion basement membrane is used as substrate for growing umbilical cord endothelium and bovine capillary endothelium. Both types of endothelial cells form continuous monolayers and do not invade the basement membrane. When human squamous carcinoma cells are inoculated on the basement/ membrane with and without the endothelium, the tumor cells attach readily to both basement membrane and the endothelial cells. The presence of either bovine or human endothelium reduced significantly the rate of tumor cell invasion. 6) Amnion basement membrane can be used as a growth substrate for normal hepatocytes. Adult rat hepatocytes isolated by *in vivo* bile duct collagenase infusion. They attach better to the amnion than any of the collagen

coated dishes, types I to V. Twice as many cells attach to the amnion as to type IV collagen which was the best collagen substrate. Once attached, the hepatocytes survived on the amnion for three weeks. Viability is tested with hematoxylin-eosin staining of the amnion membrane.

#### Significance to Biomedical Research and the Program of the Institute:

The amnion system contains an intact, human basement membrane, the most important barrier to invading tumor cells. The amnion basement membrane resembles in structure and composition subendothelial and subepithelial basement membranes. Therefore, it is an ideal model for studying in vitro the mechanisms involved in tumor cell attachment and the invasion process. When endothelium is cultivated on the amnion basement membrane, a structure is reconstituted which closely resembles a vascular wall. Numerous biological and pharmacological agents can be tested in this system and their effects on different parameters of the invasive process can be quantitatively evaluated. Furthermore, it provides means to isolate invasive cells from heterogeneous tumor cell population. This system also has a potential to be used as an assay to measure in vitro invasiveness of malignant cells from surgically removed tumors. Both human and experimental animal tumors contain admixture of tumor cells and stromal host cells. The amnion basement can be used as a barrier to sort out the invasive malignant cells. Finally this membrane can be used as a growth substrate for fastidious cells which grow poorly or not at all on plastic or other artificial surfaces.

Proposed Course of Research: a) improve the amnion invasion system by simplifying quantitation of tumor cells invading the amnion. It can be done by labelling the basement membrane of the fresh amnion or labelling tumor cells before they are placed on the membrane, b) study the effects of protein synthesis, DNA synthesis, microtubular function and cell cycle on the invasive potential of tumor cells, c) select and characterize more invasive tumor cells by repeated passage through the amnion, d) study gene expression of type IV collagenase in tumor cells, e) study further attachment, survival and function of normal hepatocytes on the amnion basement membrane.

#### Publications:

Liotta, L.A. Lee, C.W. and Morakis, D.J. New method for preparing large surfaces of intact human basement membrane for tumor invasion studies, Cancer Letters, 11: 141-152, 1980.

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Russo, R.G., Liotta, L.A., Thorgeirsson, U. and Schiffmann, E. Polymorpho-nuclear leukocyte migration through human amnion membrane. Jour. of Cell Biol. 91: 459-467, 1981.

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Russo, R.G., Foltz, C.M. and Liotta, L.A. New invasion assay using endothelial cells grown on native human basement membrane. Clinical and Experimental Metastases (in press).

Siegal, G.P., Thorgeirsson, U.P., Russo, R.G., Wallace, D.M., Liotta, L.A. and Berger S. L. Interferon enhancement of the invasive capacity of Ewing's sarcoma cells in vitro. P.N.A.S. (in press).

Effect of protease inhibitors and a chemoattractant on tumor cell invasion in vitro. Thorgeirsson, U.P., Liotta, L.A., Kalebic, T., Margulies, I.M., Thomas, K. and Russo, R.G., J.N.C.I. (in press).

Terranova, V.P., Liotta, L.A., Vasanthakumar, G., Thorgeirsson, U., Siegal, G.P. and Schiffman, E. The role of laminin in the adherence and chemotaxis of neutrophils. Minisymposium report (Mechanism of Chemotaxis), FASEB.

Liotta, L. and Thorgeirsson, U. Role of collagenases in Tumor invasion. Invited review, Tumor Invasion and Metastases, (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08258-03 LPP									
PERIOD COVERED October 1, 1981 to September 30, 1982											
TITLE OF PROJECT (80 characters or less) Tumor Desmoplasia: Characterization of the Collagens Produced by Human Tumors											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: L. Liotta</td> <td style="width: 33%;">Senior Investigator</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Others: S. Barsky</td> <td>Staff Expert</td> <td>LP, NCI</td> </tr> <tr> <td>T. Kalebic</td> <td></td> <td>LPP, NCI</td> </tr> </table>			PI: L. Liotta	Senior Investigator	LPP, NCI	Others: S. Barsky	Staff Expert	LP, NCI	T. Kalebic		LPP, NCI
PI: L. Liotta	Senior Investigator	LPP, NCI									
Others: S. Barsky	Staff Expert	LP, NCI									
T. Kalebic		LPP, NCI									
COOPERATING UNITS (if any)  Laboratory of Pathology, NCI											
LAB/BRANCH Laboratory of Pathophysiology											
SECTION Office of the Chief											
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0									
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS					
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input type="checkbox"/> (c) NEITHER									
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords)  The study is designed to biochemically characterize the collagen produced by several human tumors. Specifically the aims are to 1) investigate the types of <u>collagens</u> produced by various human tumors 2) to investigate the mechanism of <u>human breast cancer desmoplasia</u> .											



Project Description:

Objectives:

1. Characterize collagenal types found in human breast cancer desmoplasia.
2. Identification and purification of the collagens and other connective tissue proteins produced by human tumors growing in nude mice.

Methods Employed: To establish the malignant component of the tumor it is transplanted into nude mice and serially passaged. Human chondrosarcomas, osteosarcomas, or carcinomas are taken directly from the operating room when tumor cells are still viable. Tumors are then incubated with [ $^{14}$ C]proline in the presence of ascorbate, to initiate collagen synthesis, and  $\beta$ -aminopropionitril, to inhibit cross-linking. The collagen then is pH extracted using 2 methods: a) NaCl precipitation at acid and neutral and b) ion-exchange chromatography using a DEAE cellulose column with NaCl gradient. The collagen is then studied by SDS polyacrylamide gel electrophoresis employing pepsin digestion and treatment with bacterial collagenase. To establish the identity of the collagen produced, extracts are subjected to cyanogen bromide cleavage and the subsequent peptides are mapped on polyacrylamide gels. Purified extracts are then injected into rabbits and antibody is collected. Sections of tumor are studied with immunofluorescence using antibodies to types I, II, III and IV collagen. Tumors are also studied by electron microscopy.

Major Findings:

1. Desmoplasia of human breast cancer has a characteristic profile with marked increase in type V collagen.
2. The type V collagen is produced by myofibroblasts recruited by tumor associated soluble factors.
3. The chondroblastic component of a transplanted osteosarcoma produces type II collagen. Human osteosarcoma produces type I collagen. A human renal cell carcinoma a mucoepidermoid carcinoma and an angiosarcoma grown in nude mice synthesize chemical amounts of laminin.

Significance to Biomedical Research and the Program of the Institute:

Although tumor desmoplasia has been recognized for centuries, the mechanism of this phenomenon has been unknown. This work is the first to identify any biochemical differences in desmoplastic connective tissue and to develop a comprehensive theory explaining the mechanism of desmoplasia.

Proposed Course of Research: a) to correlate this type of collagen synthesized with the biologic behavior of connective tissue tumors, b) to correlate proteoglycan and glycoprotein synthesis with the biologic behavior of connective tissue tumors.

Publications:

Lanzer, W.L., Liotta, L.A., Yee, C., Azar, H., Costa, J. Synthesis of pro-collagen type II by a xenotransplanted human chondroblastic osteosarcoma. Am. J. Path. 104: 217-226, 1981.

Liotta, L. A. Tumor Extracellular Matrix. Laboratory Invest. (in press).

Barsky, S., Rao, N. C., Grotendorst, G., and Liotta, L. Increased type V collagen in desmoplasia of human breast carcinoma. Am. J. Pathol. (in press).

Dickman, P., Liotta, L. A. and Triche, T. Collagen synthesis by Ewings sarcoma. Lab. Invest. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08259-03 LPP																
PERIOD COVERED October 1, 1981 to September 30, 1982																		
TITLE OF PROJECT (80 characters or less) Interactions of metastatic tumor cells with extracellular matrix																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																		
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">L. Liotta</td> <td style="width: 20%;">Senior Investigator</td> <td style="width: 30%;">LPP, NCI</td> </tr> <tr> <td>Others:</td> <td>C. Foltz</td> <td>Research Chemist</td> <td>LPP, NCI</td> </tr> <tr> <td></td> <td>T. Kalebic</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td></td> <td>S. Barsky</td> <td>Staff Expert</td> <td>LP, NCI</td> </tr> </table>			PI:	L. Liotta	Senior Investigator	LPP, NCI	Others:	C. Foltz	Research Chemist	LPP, NCI		T. Kalebic	Visiting Fellow	LPP, NCI		S. Barsky	Staff Expert	LP, NCI
PI:	L. Liotta	Senior Investigator	LPP, NCI															
Others:	C. Foltz	Research Chemist	LPP, NCI															
	T. Kalebic	Visiting Fellow	LPP, NCI															
	S. Barsky	Staff Expert	LP, NCI															
COOPERATING UNITS (if any)																		
Laboratory of Chemistry, NIAMDD; Surgery Branch, NCI																		
LAB/BRANCH Laboratory of Pathophysiology																		
SECTION Office of the Chief																		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205																		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5																
CHECK APPROPRIATE BOX(ES)																		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER																		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																		
SUMMARY OF WORK (200 words or less - underline keywords)																		
<p>           We have already characterized and partially purified a <u>collagenase</u>, neutral metal protease produced by several metastatic tumors, which specifically degrades basement membrane (type IV) collagen. We also have correlated the metastatic potential <u>in vivo</u> of different tumor cell lines with the production of this enzyme. We are trying to obtain an <u>antiserum</u> on this enzyme to better understand the mechanism of activity on the substrate and the effective role of this collagenase in the metastatic process. We have also identified that specific <u>glycoproteins</u> mediate interactions of tumor cells to basement membrane. We have identified a new <u>receptor</u> on <u>human breast carcinoma</u> cells which mediates this interaction.         </p>																		

Project Description:

Objectives: The objectives of this project are to a) obtain an antiserum to anti-type IV collagenase b) to obtain fragments of basement membrane molecules and c) to use these factors in vivo, then to check the possibility of inhibition, in vivo and in vitro, tumor invasion and the metastatic process.

Methods Employed: Crude collagenase is obtained from serum-free cultures of highly metastatic mouse tumor (PMT sarcoma). The enzyme activity is precipitated with ammonium sulfate and purified by molecular sieve and collagen affinity chromatography. Trypsin activated or inactivated enzyme is applied to gel electrophoresis and the enzyme bands are cut out, minced and injected directly S.Q. into a rabbit. This is followed by booster injections. The specificities of the antibody are verified by immunoprecipitation. Presence of specific antibodies will be confirmed by immunodiffusion and immunofluorescence experiments on frozen sections. This last procedure is applied to specimens of human tumors obtained at surgery. In addition, the effect of the antiserum on tumor cell metastases in vivo is assayed. After checking the viability of the cells, preincubated with the antibodies, they are injected I.V. in mice to study the possible effect in retarding or preventing their metastatic capacity.

Major Findings: Collagenase activity specific for type IV collagen is already continuously obtained from mice tumors and partially purified in our lab by molecular sieve and Con A-agarose chromatography. Latent collagenase was elaborated by human breast carcinoma cells in continuous culture. Our hypothesis is that this collagenase, which degrades the major structural component of the basement membranes, is an important mechanism that allows the tumor cells to traverse this mechanical and physiological barrier and to reach first the blood circulation and later to leave it and pass on to new tissues.

A new assay has been developed in which type IV collagen is bound to a solid phase. Degradation of this substrate is then measured for living cells or enzyme applied directly to the substrate.

We have produced antibodies to the partially purified enzyme: 1) after the incubation of the enzyme with the antiserum and precipitation of the complexes, we failed to find enzymatic activity in the supernate; 2) 2 hrs preincubation of PMT sarcoma cells with the antiserum followed by their I.V. injection on to the mice, completely inhibited the formation of pulmonary metastases. The normal rabbit serum did not affect or interfere with the metastatic capacity. A breast cancer cell surface receptor has been identified which binds the basement membrane molecule laminin. A specific fragment of laminin was shown to bind to this receptor and abolish tumor cell attachment and invasion of matrix in vitro.

Significance to Biomedical Research and the Program of the Institute: Specific antibody against tumor collagenase or breast cancer cell receptor for laminin binding may be useful as a diagnostic tool when used in a radioimmunoassay.

Proposed Course of Research: The future course of this research will involve: a) purification of the immunoglobulin fraction from the antiserum; b) radioimmunoassay to quantitate the enzyme c) active immunization of animals and d) studies on the interaction antibodies-collagenase to have more information on the production and activation of the enzyme e) an in vivo study of laminin and type IV collagen fragments on metastases.

Publications:

Liotta, L.A., Goldfarb, R.F., Brundage, R., Siegal, G.P., Terranova, V., and Garbisa, S.: Effect of urokinase, plasmin and thrombin on glycoprotein and collagenous components of basement membrane. Cancer Res. 41: 4629-4630 1981.

Garbisa, S., Liotta, L.A., Tryggvason, K. and Siegal, G. Antibodies to collagenase resistant regions of type IV collagen stain whole basement membrane and cross react with 7S collagen. FEBS Letters 127: 257-262, 1981.

Siegal, G.P., Barsky, S.H., Terranova, V.P., and Liotta, L.A.: Stages of neoplastic transformation of human breast tissue as monitored by dissolution of basement membrane components. An immunoperoxidase study. Invasion and Metastasis. 1: 54-70, 1981.

Liotta, L.A., Goldfarb, R.H. and Terranova, V.P.: Cleavage of laminin by thrombin and plasmin: Alpha thrombin selectively cleaves the beta chain of laminin. Thrombosis Res. 21: 663-673, 1981.

Lanzer, W., Liotta, L.A., Yee, C., and Costa, J.: Synthesis of pro-type II collagen by a xenotransplanted human chondroblastic osteosarcoma. Am. J. Pathol. 104: 217-226, 1981.

Liotta, L.A., Terranova, V.P., Lanzer, W., Russo, R.G., Siegal, G.P. and Garbisa, S.: Basement membrane attachment and degradation by metastatic tumor cells. In H.H. Schone (Ed.): New Trends in Basement Membrane Research, in press.

Siegal, G.P., Brundage, R.A., Barsky, S.H. and Liotta, L.A.: Applications of immunoperoxidase staining to pathologic studies of human breast diseases. In Liotta, L. A., and Hart, I. R. (Eds.): New Developments in the Biology of Cancer Invasion and Metastases: Martinus Nijhoff, The Hague, in press.

Foltz, C.M., Siegal, G.P., Russo, R.G., Terranova, V.P., and Liotta, L.A.: Interactions of tumor cells with whole basement membrane in the presence or absence of endothelium. In Jamison (Ed.): Proceedings of the Cheseapeak Conference on Thrombosis and Cancer, in press, 1981.



Liotta, L.A. and Hart, I.R.: (Eds.): The Biology of Cancer Invasion and Metastases. Martinus Nijhoff, The Hague (in press), (Book with 30 chapters).

Liotta, L., Tryggvasson, K. and Garbisa, S.: Biochemical mechanisms involved in tumor cell penetration of the basement membranes. In The Biology of Cancer Invasion and Metastases. Martinus Nijhoff, The Hague (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08263-02 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Essential Fatty Acids and Mammary Gland Development and Tumorigenesis		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Barbara K. Vonderhaar                      Research Chemist                      LPP, NCI		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NIH, NCI, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (X) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project is designed to evaluate the developmental patterns of <u>mammary glands</u> of mice fed diets containing different levels of <u>saturated and unsaturated fatty acids</u> . In addition, we wished to establish an atrophied mammary gland similar to that of immature animals but in an "adult" animal. The subsequent impact of manipulation of <u>hormones</u> and dietary lipid intake on mammary tumor incidence is also to be evaluated. Studies include: 1) Examination of the morphology of mammary glands of mice at various ages after being fed EFA-deficient and control diets; 2) evaluation of the effects of various periods of EFA-deficiency on formation of <u>hyperplastic alveolar nodules</u> (HAN) and ultimately on mammary tumor formation in mice.		

## Project Description:

Major Objectives: The purpose of these studies was to define the morphologic development of mammary glands of mice placed on an essential fatty acid (EFA) deficient diet for various periods of time. Subsequent formation of hyperplastic alveolar nodules (HAN) and mammary tumors was to be assessed. The development of an atrophied mammary gland similar to that of an immature animal but in an "adult" was attempted.

Methods Employed: C3H/HeN (MMTV<sup>+</sup>) mice were used. Pregnant animals were placed on either a control or EFA deficient diet at mid-term. Female offspring were maintained on the appropriate diet.

Morphology of mammary glands was examined by hematoxylin staining of whole mounted preparations. Hyperplastic alveolar nodules (HAN) were identified and counted with the aid of a dissecting microscope. The experimental diets were similar in 95% of total composition. The remaining 5% of the control diet was composed of corn oil. The EFA-deficient diet contained 5% triglycerides resynthesized from fatty acids of 8-14 carbon chain length purified from hydrolyzed coconut oil or palm oil. The diets were checked for lipid content, lipid profile and fatty acid composition by gravimetric analysis, thin-layer, and gas-liquid chromatography respectively. Diets were obtained from 2 sources, Teklad Test Diets and Zeigler Bros.

Major Findings: C3H/HeN mice placed on the EFA-deficient diet in utero developed a severe deficiency rapidly. Of the mice on Teklad EFA deficient diet (TD) examined at 3 mon. of age, only 50% were able to survive an additional month on the diet. TD mice 8-11 wks old weighed only 50-60% of controls. This difference continued even with those few TD mice able to survive 8 mon. Even at 3 mon. of age TD animals' body fat was nearly non-existent. Whole mounts of the glands of these animals showed the epithelial component of 3 mon. old TD mice consists of a short outgrowth from the nipple with little ductal branching and no alveoli. At most, this outgrowth fills 1/4 of the sparse fat pad and resembled an immature gland. In contrast, the fat pad of the 3-4 mon. old control (TC) animal is completely filled with highly branched ducts and frequent alveoli.

However, analysis of the Teklad EFA-deficient diet showed that it did not contain clearly defined short chained fatty acids (C8-14) and appeared to be too low in total fat (only 3.5 to 4.4%). Therefore, EFA deficient (ZD) and corn oil control (ZC) diets were obtained from Zeigler Bros. These diets were analyzed and found to have 4.5 to 5% fat as expected. However, female mice raised on the Zeigler deficient diet since in utero were much healthier than TD animals at 3 mon. of age. Their body weight was 75-80% of controls at 3 months of age. They were able to survive beyond 6 mon. of age. Their hair showed a slight roughness and reddishness indicating an EFA-deficiency. Their body fat at 6 mon. of age was significantly less than that of ZC by visual appraisal. Whole mounts of the glands of 3 mon. old ZD mice showed ductal expansion throughout the fat pad. The ducts were sparse with little branching and no alveoli. While the glands were deficient they were not "atrophied" and did not resemble an immature gland.

One major difference in the TD and ZD diets was the myristic acid content. while TD was very deficient (trace amounts) in myristic acid, it was relatively

high (17.53% of total fatty acids) in ZD. Since myristic acid has been shown to promote dome formation in cultured rat mammary cells, myristic acid pellets were implanted in 3 mon. old TD animals to see if it promoted growth of the mammary epithelium. It did not.

A Ziegler EFA deficient diet also low in myristic acid was then obtained by substituting hydrolyzed coconut oil with palm oil (ZP). This diet resulted in glands in 3 mon. old mice similar to those found in ZD animals. An "immature" gland in an adult animal was not achieved.

Significance to Biomedical Research and the Program of the Institute:

Epidemiological studies have shown a strong positive correlation between per capita fat consumption and the incidence of human breast cancer in various countries. Elevated dietary fat has been shown to facilitate development of both spontaneous and carcinogen-induced mammary tumors in rodents. Elevated dietary lipids have been shown to alter prolactin secretion and clearance in rodents. Prolactin may play a key role in initiation of mammary tumorigenesis. In addition unsaturated fats may act directly on the mammary gland to affect the growth, development, and hormonal responsiveness of the epithelial cells. In the U.S. recent studies have indicated that while the level of consumption of animal fat has been decreasing, increased consumption of vegetable fat, high in unsaturated fatty acids, has occurred. Thus we designed studies to help to understand the impact of the unsaturated, essential fatty acids on mammary development, hormonal sensitivity and subsequent tumorigenesis.

Proposed Course of Research: The major purpose of this project was to produce an "atrophied" or immature mammary gland in an adult animal. This could only be achieved with the Teklad EFA deficient diet which was not a well defined diet. A Ziegler EFA deficient diet resulted in underdeveloped but not "immature" glands. Thus, we could not achieve the initial purpose of this study. This project is neither cost-effective nor manpower effective for the type of results obtained and thus has been discontinued. A brief report of our significant observations will be written up for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF Project INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  No. Z01 CB 08264-02 LPP						
PERIOD COVERED <u>October 1, 1981 to September 30, 1982</u>								
TITLE OF PROJECT (80 characters or less) <u>Cyclic Nucleotide and Carcinogenesis</u>								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: H. Hasuma</td> <td style="width: 33%;">Visiting Fellow</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Other: Y.S. Cho-Chung</td> <td>Chief, Cellular Biochemistry Sec.</td> <td>LPP, NCI</td> </tr> </table>			PI: H. Hasuma	Visiting Fellow	LPP, NCI	Other: Y.S. Cho-Chung	Chief, Cellular Biochemistry Sec.	LPP, NCI
PI: H. Hasuma	Visiting Fellow	LPP, NCI						
Other: Y.S. Cho-Chung	Chief, Cellular Biochemistry Sec.	LPP, NCI						
COOPERATING UNITS (if any)								
LAB/BRANCH <u>Laboratory of Pathophysiology</u>								
SECTION <u>Cellular Biochemistry Section</u>								
INSTITUTE AND LOCATION <u>NIH NCI, Bethesda, MD 20205</u>								
TOTAL MANYEARS: <u>1.25</u>	PROFESSIONAL: <u>1.25</u>	OTHER: <u>0</u>						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords)  <p>           Carcinogenic doses of <u>7,12-dimethylbenz(α)anthracene (DMBA)</u> failed to induce <u>mammary carcinomas</u> in rats receiving <u>dibutyryl cyclic AMP (DBcAMP)</u>. The <u>inhibitory effect</u> of DBcAMP on the <u>mammary carcinogenesis</u> correlated with the antagonistic action of DBcAMP on the <u>DNA-binding</u> of DMBA in vivo. The antagonistic interrelation between DBcAMP and DMBA in their DNA-binding was also demonstrable with <u>mammary epithelial cells</u> in culture. The results suggest that DBcAMP may prevent the induction of mammary carcinogenesis by blocking the binding to DNA of the carcinogen.         </p>								



Project Description:

Methods Employed:

- i. DMBA intubation.  
Sprague-Dawley female rats (50 days old) were given a single intubation of 7,12-dimethylbenz( $\alpha$ )anthracene (DMBA) (20mg in 1 ml sesame oil).
- ii. DBcAMP administration.  
DBcAMP (10 mg/200g rat/day), beginning 1 day prior to DMBA intubation.
- iii. Cyclic AMP level: intracellular cAMP level was measured by the radio-immunoassay using the acetylation procedure exactly as described by Collaborative Research Inc.
- iv. Adenylate cyclase assay: Adenylate cyclase activity was measured by the method of Krishna et al.
- v. Protein kinase assay: The kinase activity was measured by measurement of the incorporation of  $^{33}\text{P}$  from  $\gamma$ -labeled ATP into histones +  $10^{-6}\text{M}$  cAMP.
- vi. DNA isolation: DNA was isolated following the method of Huberman and Sacks.
- vii. Mammary epithelial cells: mammary epithelial cells obtained by collagenase digestion of mammary gland were grown in culture and only duct cells were used in the experiments.

Major Findings

- I. Inhibitory effect of DBcAMP on DNA-binding of DMBA in mammary gland.
  1. In vivo, a marked decrease in the covalent binding of [ $^3\text{H}$ ]DMBA to mammary gland DNA was observed after oral administration of DBcAMP (10 mg/200g rat) starting 1 day prior to [ $^3\text{H}$ ]DMBA intubation.
  2. The DNA-binding of [ $^3\text{H}$ ]DMBA accompanied changes in the cAMP system of the mammary gland: the intracellular cAMP level and adenylate cyclase activity increased and cAMP-dependent protein kinase type I newly appeared. Only type II cAMP-dependent protein kinase was detected in the control mammary gland (no DMBA intubation).
  3. In mammary glands of rats receiving DBcAMP the cAMP system became similar to that of older rats that are no longer susceptible to the carcinogen.
  4. The antagonistic interrelation between DBcAMP and DMBA in their DNA-binding was also demonstrable in vitro. Incubation of the mammary gland slices at  $30^\circ\text{C}$  with either [ $^3\text{H}$ ]DMBA ( $10^{-6}\text{M}$ ) or [ $^3\text{H}$ ]DBcAMP ( $10^{-6}\text{M}$ ) showed a time dependent incorporation of the radioactivity into DNA, respectively. The presence of unlabeled DBcAMP ( $10^{-5}\text{M}$ ) inhibited the DNA-binding of [ $^3\text{H}$ ]DMBA by 50%, and the presence of unlabeled DMBA ( $10^{-5}\text{M}$ ) inhibited the DNA-binding of [ $^3\text{H}$ ]DBcAMP by 60%.

## II. Antagonism between DBcAMP and DMBA in their DNA-binding in mammary epithelial cells in culture.

1. Mammary epithelial duct cells growing in culture exhibited a time dependent uptake of radioactivity into their DNA when incubated with either [ $^3\text{H}$ ]DBcAMP or [ $^3\text{H}$ ]DMBA. The DNA-binding of [ $^3\text{H}$ ]DMBA was maximal in the mammary epithelial cells of 50 day old rats and was minimal in both 30 and 120 day old rats. The DNA-binding of [ $^3\text{H}$ ]DBcAMP was, however, increased with the age of rats.
2. The DNA-binding of [ $^3\text{H}$ ]DMBA ( $10^{-6}\text{M}$ ) was inhibited in the presence of unlabeled DBcAMP ( $10^{-8}\text{M} \sim 10^{-5}\text{M}$ ), the maximum inhibition of 50% was observed at  $10^{-6}\text{M}$  DBcAMP.
3. The DNA-binding of [ $^3\text{H}$ ]DBcAMP ( $10^{-6}\text{M}$ ) was inhibited in the presence of unlabelled DMBA ( $10^{-8}\text{M} \sim 10^{-5}\text{M}$ ), the maximum inhibition of 50% was observed at  $10^{-6}\text{M}$  DMBA.
4. The antagonism between DMBA and DBcAMP in their DNA-binding was only evident in the mammary epithelial cells of age 50 rats. These results suggest that DBcAMP may prevent the induction of mammary carcinomas by blocking the binding to DNA of the carcinogen.

Significance to Cancer Research and the Program of the Institute: (NCP Objective #6, approach #3) These studies contribute to the understanding of the molecular mechanism of carcinogenesis. The results suggest the antagonistic effect of cAMP at the DNA-binding of the carcinogen. The suppression of mammary carcinogenesis found with orally administered DBcAMP may be of significance in the prevention studies of mammary cancer in humans.

Proposed Course of Research: To extend the investigation on the mechanism of the role of cAMP in mammary carcinogenesis, the following proposals are made: 1) Assess the subcellular localization of [ $^{14}\text{C}$ ]DMBA and [ $^3\text{H}$ ]DBcAMP following their administration in vivo and in vitro to detect the site(s) of their antagonistic action, 2) Examine DNA-adducts following the administration of DMBA + DBcAMP 3) Assess whether phosphorylation of plasma membranes or nuclear proteins takes place with DMBA + DBcAMP incubation. 4) Assess the role of cAMP-dependent protein kinase type I & II in DMBA-carcinogenesis. 5) Examine the relationship between DMBA-carcinogenesis and gene modulation using in vitro protein synthesizing systems.

### Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08265-02 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Isolation and Characterization of a Type V Collagenolytic Enzyme		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;">           PI: Lance Liotta             Others: T. Kalebic         </div> <div style="width: 45%;">           Senior Investigator             Visiting Fellow         </div> <div style="width: 10%; text-align: right;">           LPP, NCI         </div> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input checked="" type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div> <input type="checkbox"/> (a1) MINORS         </div> <div> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)  A neutral metal protease has been identified which cleaves <u>native type V collagen</u> under conditions where pepsinized type IV collagen or the interstitial collagens are not significantly degraded. The enzyme is secreted into the media of cultured <u>M50-76 reticulum cell sarcoma</u> (malignant macrophages) and <u>leiomyosarcoma tumor cells</u> . Biosynthetically labeled type V collagen prepared from organ cultures of human amnion membrane is used for a routine assay of type V collagenolytic activity. The partially purified enzyme a) exists in a latent form requiring trypsin activation for maximum activity; b) has a molecular weight estimated by molecular sieve chromatography of approximately 80,000 daltons; c) is inhibited by EDTA but not phenylmethylsulfonyl fluoride; and d) produces specific cleavage products of both A and B collagen chains.		

Project Description:

Objectives: To identify and characterize collagenolytic activity for type V collagen and to purify this enzyme.

Methods: Serum free culture media reticulum cell sarcoma and a human leiomyosarcoma is precipitated with ammonium sulfate and the resultant proteins are dialyzed and chromatographed on a molecular sieve column and further purified by high pressure liquid chromatography. Enzyme activity is assayed using polyacrylamide gel electrophoresis of cold type V collagen and trichloroacetic acid/tannic acid precipitation of  $^{14}\text{C}$  labeled type V collagen digestion products. Further studies of purified enzyme are performed using isoelectric focusing.

Major Findings: Type V collagenolytic metalloproteinase was partially purified from the media of cultured reticulum cell sarcoma cells, as described previously, using ammonium sulfate precipitation, molecular sieve chromatography and high pressure liquid chromatography. The enzyme appears as a doublet of 80000 Mr on polyacrylamide gel electrophoresis and has an isoelectric point of pH 7.5

The protease susceptibilities of recently identified cartilage collagens HMW,  $1\alpha$ ,  $2\alpha$ , and  $3\alpha$  were investigated. Mammalian skin collagenase cleaved the  $3\alpha$  chain under conditions where HMW,  $1\alpha$  and  $2\alpha$  were not degraded. A tumor cell derived type V collagenolytic metalloproteinase degraded HMW,  $1\alpha$  and  $2\alpha$ , but not  $3\alpha$ . Plasmin or leucocyte elastase failed to significantly degrade any of the cartilage collagens when digestion was performed at  $25^\circ\text{C}$  (15 hours, enzyme to substrate ratio 1:100). At  $36^\circ\text{C}$  but not  $33^\circ\text{C}$   $\alpha$  thrombin degraded HMW,  $1\alpha$  and  $2\alpha$ , with little or no degradation of  $3\alpha$ . This pattern of protease susceptibility for HMW,  $1\alpha$  and  $2\alpha$  is therefore similar to type V collagen. The cleavage of  $3\alpha$  by skin collagenase but not by elastase is similar to type II collagen. These results suggest that HMW,  $1\alpha$  and  $2\alpha$  are part of the type V collagen family.

Significance to Biomedical Research and the Program of the Institute: A type V collagenolytic enzyme has been identified and purified for the first time. Since type V collagen may be pericellular in location, cell migration may be associated with type V collagen turnover. Moreover, the presence of a type V degrading enzyme in tumor cells may in part have some influence on these cells ability to invade tissues and on their metastatic organ distribution.

Proposed Course of Research: a) To develop antibodies to the type V collagenase to see if this activity can be inhibited, b) to survey other tumor cell lines for this enzymatic activity, c) to further characterize and purify this enzyme with other biochemical methods, d) to utilize this enzyme in the study of the molecular structure of type V collagens.

Publications:

Liotta, L.A., Lanzer, W.R. and Garbisa, S.: Identification of a type V collagenolytic activity. Biochem. Biophys. Res. Comm., vol. 98, no. 1, 1981.

Liotta, L.A., Kalebic, T., Reese, C.A. Mayne, R. Protease susceptibilities of HMU,  $1\alpha$ ,  $2\alpha$ , but not  $3\alpha$  cartilage collagens are similar to type E collagen. Biochem. Biophys. Res. Commun., Vol. 104, No. 2, 1982.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08266-02 LPP															
PERIOD COVERED October 1, 1981 to September 30, 1982																	
TITLE OF PROJECT (80 characters or less)  Structure and Biological Function of Basement Molecules.																	
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																	
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Lance Liotta</td> <td style="width: 33%;">Senior Investigator</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Others: Nagasawara Rao</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td>Inger Margulies</td> <td>Biologist</td> <td>LP, NCI</td> </tr> <tr> <td>Victor Terranova</td> <td>Senior Investigator</td> <td>D, LDBA</td> </tr> <tr> <td>S. Barsky</td> <td>Staff Expert</td> <td>LP, NCI</td> </tr> </table>			PI: Lance Liotta	Senior Investigator	LPP, NCI	Others: Nagasawara Rao	Visiting Fellow	LPP, NCI	Inger Margulies	Biologist	LP, NCI	Victor Terranova	Senior Investigator	D, LDBA	S. Barsky	Staff Expert	LP, NCI
PI: Lance Liotta	Senior Investigator	LPP, NCI															
Others: Nagasawara Rao	Visiting Fellow	LPP, NCI															
Inger Margulies	Biologist	LP, NCI															
Victor Terranova	Senior Investigator	D, LDBA															
S. Barsky	Staff Expert	LP, NCI															
COOPERATING UNITS (if any) NIDR, Laboratory of Developmental Biology & Anamolies																	
<del>Lab</del> Laboratory of Pathophysiology																	
<del>Office</del> Office of the Chief																	
INSTITUTE AND LOCATION, Maryland 20205																	
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.0	OTHER: 0.5															
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																	
SUMMARY OF WORK (200 words or less - underline keywords)  Highly purified $\alpha$ -thrombin, plasmin, cathepsin G, chymotrypsin, trypsin and urokinase were incubated with laminin, type IV collagen and type V collagen. At 25°C (1:100 enzyme to substrate ratio on a weight/weight basis) $\alpha$ -thrombin selectively degraded the $\beta$ chain of the native laminin, whereas plasmin cathepsin G, trypsin and chymotrypsin degraded both the $\alpha$ and $\beta$ chains. The specific limited cleavage fragments of laminin produced by $\alpha$ -thrombin and the other enzymes were purified by HPLC, studied <u>electron microscopy</u> and evaluated for biologic activity. A model was proposed for the <u>structure of laminin</u> and which domains of the molecule mediate various biologic functions. A cell <u>receptor</u> for laminin was identified.																	

Objectives:

1. To determine the susceptibility of native laminin to degradation by serine proteases.
2. To use the degradation products of laminin by these proteases to further study the structure and biological function of laminin.

Project Description:

Methods: Highly purified  $\alpha$  thrombin, urokinase or plasmin were incubated at 35°C M 25°C for varying time periods with laminin, type V and type IV collagen. Laminin was extracted from EHS mouse sarcoma tissue by salt extraction and DEAE cellulose and agarose B50 Gel-A-5m column chromatography. Purified laminin was also iodinated with I<sup>125</sup> and digestion products were run on polyacrylamide gel electrophoresis. Digestion products were also run on a B10 Gel-A-1.5 column in order to separate by molecular weights digestion products.

Major Findings: The two subunits of laminin significantly differ in their need for the reducing agent (DTT) for complete migration on polyacrylamide gels. Under optimal reducing conditions, a constant ratio of 2:1 was obtained for the 400 KD ( $\beta$ subunit) and the 200 KD ( $\alpha$ subunit) chains. Of the enzymes investigated human  $\alpha$  thrombin, specifically and completely depleted the  $\beta$  subunit leaving the  $\alpha$  subunit unaltered in size and quantity. Utilizing high pressure liquid chromatography the  $\alpha$  subunit was purified and obtained in native form. Electron microscopic studies of laminin and isolated subunit enabled us to identify that the long arm measuring 78 nm represent the 400 KD chain and the  $\alpha$ -subunit consists of three similar 200 KD chains. Cell attachment data on the whole laminin,  $\alpha$  subunit, and pepsin fragment of laminin (or of the  $\alpha$  subunit) enabled us to conclude that the terminal globular domains on the small arms are required for the tumor cell to attach to IV collagen. Studies on the degradation of laminin by  $\alpha$ -thrombin, plasmin, trypsin, chymotrypsin and cathepsin G gave us the conclusion that the  $\beta$  subunit is highly protease labile and all these enzymes (except  $\alpha$  thrombin) degraded the  $\alpha$  subunit and produced a similar fragment. This fragment was also purified by HPLC and found to include the intersection of the three short arms (28 nm). We also found that this fragment specifically binds to the tumor cell surface and does not mediate the binding and actually inhibited the attachment process to IV collagen. Our studies have also showed that some of the metastatic cell lines preferentially in laminin for the attachment process. Our recent data indicated that the tumor cell surface has a receptor like moiety for laminin.

Significance to Biomedical Research and the Program of the Institute:

(1) Degradation of laminin may play a role in endothelial migration during wound healing or vascularization since migration may involve a series of sequential binding events. (2) Degradation of laminin may also facilitate passage of: immune cells and tumor cells through basement membranes. (3) Using these degradation products will allow study of the structure of laminin and may produce antibodies to various components to the molecule.

Proposed Course of Research: (1) To separate the chains by column chromatography after selective cleavage. (2) To use antibodies against these separated cleavage products to study the structure of laminin and determine where the attachment site is located. (3) To produce antibodies to the laminin attachment site and investigate it. Tumor cell attachment can be blocked by these antibodies.

Publications:

Liotta, L.A., Goldfarb, R., Terranova, V.: Cleavage of laminin by thrombin and plasmin: Alpha thrombin selectively cleaves the beta chain of laminin. Thrombosis Res. 21: 663-673, 1981.

Terranova, V. P., Liotta, L. A., Russo, R. G. and Martin, G. R.: Role of laminin in the attachment and metastasis of murine tumor cells. Cancer Res. 42: 2265-2269, 1981.

Nageswarro, C., Margulies, I.M.K., Traalba, T. S., Terranova, V. P., Madri, J. A. and Liotta, L. A.: Isolation of a subunit of laminin and its role in molecular structure and tumor cell attachment. (in press)

Nageswarao, C., Margulies, I.M.K., Goldfarb, R. H., Madri, J. A., Woodley, O.T. and Liotta, L. A.: Differential proteolytic susceptibility of laminin alpha and beta subunits. (Submitted for publication)

Terranova, V. P., Rao, C. N., Kalebic, T. and Liotta, L.: Identification of a receptor for laminin on human breast carcinoma cells. (Submitted for publication).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08268-01 LPP									
PERIOD COVERED October 1, 1981 to September 30, 1982											
TITLE OF PROJECT (80 characters or less)  Structure, topology, and dynamics of tight junctions											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT											
<table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: P. Pinto da Silva,</td> <td style="width: 40%;">Chief, Membrane Biology Sec.</td> <td style="width: 20%;">LPP, NCI</td> </tr> <tr> <td>Others: G. Tadvalkar</td> <td></td> <td>LPP, NCI</td> </tr> <tr> <td>J. Chevalier</td> <td></td> <td>LPP, NCI</td> </tr> </table>			PI: P. Pinto da Silva,	Chief, Membrane Biology Sec.	LPP, NCI	Others: G. Tadvalkar		LPP, NCI	J. Chevalier		LPP, NCI
PI: P. Pinto da Silva,	Chief, Membrane Biology Sec.	LPP, NCI									
Others: G. Tadvalkar		LPP, NCI									
J. Chevalier		LPP, NCI									
COOPERATING UNITS (if any) B. Kachar, Lab. of Neuropathology and Neuroanatomy, N.N.C.D.S., J. Chevalier and J. Bourguet, Lab. of Biomembranes, Dept. of Biology, Nuclear Research Center, Saclay, France											
LAB/BRANCH Laboratory of Pathophysiology											
SECTION Membrane Biology Section											
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205											
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:									
CHECK APPROPRIATE BOX(ES)											
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER											
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords)											
Analysis of previous thin section and freeze-fracture observations of the tight junction (Zonula occludens) lead us to propose that the junctional strands are comprised of <u>cylindrical, intramembranes inverted micelles</u> . We propose that the exoplasmic halves of plasma membranes are fused into single leaflets that assure the semipermeability properties of the junction. We show that in various epithelia massive, rapid proliferation of tight junction strands is easily accomplished. In addition, we use toad bladder, a system amenable to physiological monitoring of transport functions, where we demonstrate that proliferation of junctional strands occurs after an osmotic shock. We studied the <u>time course</u> of this proliferation, the effect of cytoskeletal perturbations and the role of calcium ions. Application of morphometric techniques and statistical analysis provides time courses for assembly and also disassembly of the junctional strands.											



Methods:

Tissues (prostate, ileum among others) are excised from lightly anesthetized rats, minced and incubated in various media at 37°C for periods ranging from 2 minutes to one hour. The tissues are then fixed in glutaraldehyde, impregnated in 30% glycerol, frozen by immersion in the liquid phase of partially solidified freon 22 and freeze-fractured. After platinum/carbon replication, cleaned replicas are observed with an electron microscope. For studies of tight junction proliferation in toad bladders, paired urinary bladders of *Bufo marinus* are mounted as sacs at the tip of a glass canula, according to the technique of Bentley. The serosal and mucosal faces of the tissue were bathed with an aerated amphibian Ringer solution (A6 Ringer containing 5 mM glucose). Osmotic shock was induced by immersing the hemibladders in distilled water for different periods of time (2, 5, 10, 30, 60 min) at room temperature or 37°C. In some cases, specimens were preincubated, before the application of the osmotic shock, with, in the serosal compartment, cycloheximide (200 ug/ml, 30 min R.T. and 5 min 37°C) or cytoskeleton perturbors (colchicine 10-3 M, 2 hr R.T. followed by colchicine 10-3 M + cytochalasin B 100 ug/ml, 2 hr R.T.) or Ringer free  $\text{Ca}^{++}$ , free  $\text{Mg}^{++}$  + EGTA 2mM (90 min R.T.). In other circumstances, after osmotic shock, the initial serosal (A6 Ringer + 5mM glucose) was restored and the tissue incubated for various period of time (30 min to 3 hr). Epithelial cells were scrapped, fixed in Ringer/glutaraldehyde 2% solution and processed for freeze-fracture.

Major Findings:

Epithelial cells are linked together, at their apical pole, by well developed intercellular contacts, the zonula occludens or tight junction, forming a continuous belt around the cells. Recent works have been devoted to study their composition and structure. These intercellular contacts are not static structure since some physiological and pharmacological treatments can induce strong modifications in the pattern of the junctional elements. Until our studies the tight junction strand was viewed as comprised by a linear assemblage of integral membrane proteins that, upon glutaraldehyde fixation, fused laterally leading to the formation of cylindrical structures generally observed in freeze-fractured epithelia. We analyzed previous studies and showed that, instead, the tight junction principal elements are inverted, cylindrical intramembranous lipidic micelles. We proposed that the tight junction represents a stable intermediate of a process of membrane fusion. In addition, we showed that, at the junctional sites, the exoplasmic halves of the fused epithelial cells are continuous, thus providing a structural basis for accounting the known permeability properties of this junction. We show that proliferation of tight junction strands occurs very rapidly (in less than 2 minutes) and even in the presence of metabolic poisons or of inhibitors of protein synthesis (cycloheximide). These results are consistent with our proposal that an inverted cylindrical micelle is a structural membrane intermediate. We discovered that an osmotic shock applied to the basal side of toad urinary bladder epithelial cells induces a massive, fast and, in certain extent, reversible proliferation of tight junction strands in an area (the basal membrane of epithelial cells) where normally, no tight junction exists. This process is very fast, temperature dependent and reaches a maximum where almost 60% of the epithelial cells develop junctional strands at their basal pole. The time-course follows a logarithmic curve with a half-time of 5 min at R.T and less than 2 min at 37°C. When developed, the strands appeared to be fairly stable structure. However, 20 to 30% of these



new formed junctional strands disappear if the tissue is again incubated in full Ringer over 2 hours at room temperature. Cycloheximide (which inhibits the protein synthesis) and cytoskeleton perturbers (colchicine and cytochalasin B) do not affect the development of tight junctions in this tissue. Cycloheximide, however, increases the rate and extent of strand disappearance. The proliferation process is achieved without any noticeable decrease in the density of the intramembraneous particles or modification of the apical zonula occludens. These results suggest that the new tight junction elements observed at the basal pole of toad bladder epithelial cells are directly formed by components already located in the plasma membrane. We also studied the effect of osmotic shock on tissues previously incubated in absence of  $\text{Ca}^{++}$  and  $\text{Mg}^{++}$ . We observed that, beside the specific effect of this preincubation on the apical zonula occludens (disruption of the meshwork of the tight junction, strand proliferation on the lateral membranes), the absence of divalent cations does not inhibit the formation under osmotic shock of new strands in the basal area of the cells.

Significance to Biomedical Research: The zonula occludens plays a major role in the structure and functions of epithelial tissues, binding cells together at their apical pole and sealing the intercellular spaces. Our proposal that inverted cylindrical micelles are principal elements of tight junction structure and that at the junctional site the exoplasmic halves of the plasma membrane are continuous is a radical departure from previous concepts, has important physiological implications, demonstrates the importance of non bilayer lipid configurations in biological membranes and suggests numerous avenues of experimentation. It has been extremely well received after its publication as a major review article in *Cell*. Proliferation of tight junction strands is an interesting phenomenon that explains previous instances reported in studies of cellular pathology (the effect of ionizing radiation, for instance). The ability of osmotic shock to induce a massive proliferation of new junctional strands in the basal part of toad bladder epithelial cells, without affecting the apical zonula occludens, provides a useful system that is amenable to physiological study (e.g. water transport) and where the genesis and the dynamic as well as the composition and structure of tight junction strands can be now investigated.

Proposed Course of Research: Amphibian epithelia are currently used as biomodels to study membrane transport and hormonal action. Their macroscopic dimension, structural simplicity, convenience of use and "in vitro" reactivity to hormonal stimuli make them attractive experimental systems. In collaboration with the Laboratory of Biomembranes, Department of Biology, Nuclear Research Center, Saclay, France, we intend to investigate the consequences of osmotic shock and tight junction proliferation on the water permeability properties of toad urinary bladder. In addition, we will pursue investigation of the structure of tight junctions in various other epithelia, in particular the identification of junctional lipids and proteins.

#### Publications:

Pinto da Silva, P. and Kachar, B.: On tight junction structure. *Cell* 28, 441-450, 1982.

Kachar, B. and Pinto da Silva, P.: Rapid, massive assembly of tight junction strands. *Science* 213, 541-544, 1981.

Chevalier, J., Tadvalkar, G., and Pinto da Silva, P.: Massive fast assembly of tight junction strands in toad bladder induced by an osmotic shock: time course study; influence of calcium and of cytoskeletal perturbors. Journal of Cell Biology, submitted for publication.

ITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08269-01 LPP						
PERIOD COVERED October 1, 1981 to September 30, 1982								
TITLE OF PROJECT (80 characters or less) Membrane differentiation: Role of integral components in membrane domains								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT								
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: P. Pinto da Silva,</td> <td style="width: 33%;">Chief, Membrane Biology Sec.</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Others: A.P. Aguas</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> </table>			PI: P. Pinto da Silva,	Chief, Membrane Biology Sec.	LPP, NCI	Others: A.P. Aguas	Visiting Fellow	LPP, NCI
PI: P. Pinto da Silva,	Chief, Membrane Biology Sec.	LPP, NCI						
Others: A.P. Aguas	Visiting Fellow	LPP, NCI						
COOPERATING UNITS (if any)								
LAB/BRANCH Laboratory of Pathophysiology								
SECTION Membrane Biology Section								
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205								
TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.7	OTHER: 0.1						
CHECK APPROPRIATE BOX(ES)								
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER								
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords) The topology of <u>sialoglycoconjugates</u> (SGCs) and <u>mannose/glucose glyco-</u> <u>components</u> (M/GCs) was studied in two plasma <u>membrane domains</u> of polarized cells. SGCs and M/GCs were localized <u>in situ</u> , by WGA and Con A labeling respectively, in four membrane planes: external and cytoplasmic surfaces; exoplasmic and endoplasmic faces of freeze-dissected membranes. SGCs and M/GCs are asymmetrically inserted in the plasma membrane, being preferenti- ally associated with outer membrane halves of the studied cells; correlation between SGCs and membrane-intercalated particles densities is observed in protoplasmic halves of the membrane domains, thus suggesting that membrane domains (defined by surface probes) may be conditioned by integral mem- brane components; ' <u>fracture-label</u> ' allows the detection of SGCs and M/GCs expressed at the external surface that are masked by the cell coat and are not accessible to surface probes in intact cells.								

Methods:

Inner and outer membrane surfaces and fracture-faces of membrane halves of plasma and acrosomal membranes of spermatozoa from different species were made accessible to Con A and WGA labeling (lectin-colloidal gold conjugates) upon osmotic shock or 'fracture-label.' In some samples, surface Con A and WGA binding-sites were immobilized prior to freeze-fracture by cell incubation with Con A-ferritin or WGA-ferritin. The specimens were processed for conventional thin-section, freeze-fracture, and freeze-fracture cytochemistry. The relative density of lectin receptors associated with the surfaces and fracture-faces of the membrane domains of spermatozoa was evaluated by comparing the distribution of colloidal gold granules detected on the different membrane areas.

Major Findings:

- 1) SGCs and M/GCs are asymmetric inserted in the plasma membrane of spermatozoa, being preferentially associated with the outer membrane half.
- 2) In membrane halves, regionalization of SGCs is restricted to topoplasmic membrane halves. This indicates that membrane surface domains are conditioned by integral membrane components.
- 3) Acrosomal membrane show Con A and WGA binding sites on both surfaces of the acrosomal membrane, thus being an exception to the radical membrane asymmetric of carbohydrates observed in most biological membranes.

Significance to Biomedical Research:

The topographical segregation of specific surface molecules in differentiated cells is frequently correlated with the performance of specific function. For example, acetylcholine receptors of muscle cells are concentrated on the plasma membrane at points of innervation, low density lipoprotein receptors are restricted to coated pits of fibroblasts, clustering of lectin receptors has been reported in transformed cells and related with agglutinability changes. We plan to study the structural characteristics involved in the formation, stability, and dynamics of membrane domains components. For now, the mammalian spermatozoa was chosen as the elective model to study these phenomena because of the high polarity of the cell and of the good characterization of its membrane domains.

Proposed Course of Research:

- 1) Use of different classes of antisperm monoclonal antibodies (to be provided by Drs. Myles and Primakoff) in the definition of the topology of sperm antigens.
- 2) Dynamics of membrane domain components during membrane disassembly and fusion.

Publications:

Aguas, A.P. and Pinto da Silva, P. Topology of sialoglycocomponents in plasma in plasma membrane domains of a polarized cell: Freeze-Fracture cytochemistry of boar spermatozoa. Science, submitted for publication.

Aguas, A.P. and Pinto da Silva, P. Symmetry of membrane carbohydrates: Localization of Concanavalin A and Wheat-germ Agglutinin in intact and freeze-fractured acrosomal membranes. Nature, submitted for publication.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08270-01 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Intracellular compartmentalization of membrane glycoproteins		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: P. Pinto da Silva Others: M.R. Torrissi C. Parkison	Chief, Membrane Biol. Sec. Visiting Fellow Chemist	LPP, NCI LPP, NCI LPP, NCI
COOPERATING UNITS (if any)		
LAB/BRANCH <u>Laboratory of Pathophysiology</u>		
SECTION <u>Membrane Biology Section</u>		
INSTITUTE AND LOCATION <u>NCI, NIH Bethesda, MD 20205</u>		
TOTAL MANYEARS: 0.9	PROFESSIONAL: 0.6	OTHER: .2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) New " <u>fracture-label</u> " techniques recently developed in our laboratory are used to show the distribution and partition of glycoproteins in <u>intracellular</u> membranes of secretory as well as non-secretory cells. We define the existence of two membrane types: Those with fully glycosylated <u>sialoglycoproteins</u> are intensely labelled by wheat germ agglutinin/colloidal gold complexes; other membranes, which are not labelled by WGA but are intensely labelled by Concavalin A/colloidal gold and their (i.e. their glycoproteins are not sialidated). Our study shows, for the first time and at high resolution (better than 20 nm) the pattern of intracellular distribution of <u>membrane glycoproteins</u> in eukaryotic cells.		

Materials and Methods:

**Cells:** Pancreas, liver, ileum tissues, adrenal and salivary glands were exercised from adult Sprague-Dawley rats. Isolated hepatocytes and pancreatic cells were obtained by digestion of liver and pancreas tissues with collagenase type IV. Salivary and adrenal cells were isolated with collagenase type II (5 mg/ml of collagenase in Hank's solution, from Worthington Biochemical Corp., Freehold for 5-10 min. at 37°C. The isolated cells and tissues were washed twice in Hank's solution fixed in 1% - 2% glutaraldehyde in phosphate buffered saline pH 7.4 for 2-3 hours at 4°C, impregnated in 30% glycerol and frozen in Freon 22 cooled by liquid nitrogen.

Human peripheral blood lymphocytes and monocytes were isolated from fresh heparinized blood by Ficoll-Hypaque gradients, washed twice in phosphate buffered saline and fixed in 1% glutaraldehyde in the same buffer for 2 hours at 25°C. Human neutrophils were obtained by isolation in Ficoll-Hypaque gradients as above, followed by Plasmagel (Roger-Bellon Paris) sedimentation and lysis of the residual erythrocytes with Tris buffered ammonium chloride. The isolated neutrophils were washed twice in 0.1 M sodium cacodylate-HCl buffer and fixed in 1.5% glutaraldehyde in the same buffer for two hours at 25°C. Some were also incubated for 30-45 min. at 25°C in Karnovsky diaminobenzidine saturated solution containing  $H_2O_2$  as substrate (for the peroxidase reaction). All cells were impregnated in glycerol and frozen as above.

**Freeze-fracture and cytochemical label:** Frozen cells and tissues were immersed in liquid nitrogen and finely crushed with a glass pestle precooled in liquid nitrogen. The freeze-crushed with a glass pestle precooled in liquid nitrogen. The freeze-fractured cells were then thawed in 1% glutaraldehyde, 30% glycerol in phosphate buffered saline pH 7.4 and deglycerinated in 1M glycylglycine in the same buffer. Freeze fractured cells and tissues were incubated in solutions of WGA (0.25-1 mg/ml in 0.1 M Sorensen phosphate buffer, 4% polyvinylpyrrolidone pH 7.4) for 1 hr. at 37°C. Controls were pre-incubated in 0.4M N-acetyl-D-glucosamine for 15 min at 37°C and then treated with WGA in presence of the sugar for 1 hr. at 37°C. All the samples were incubated in the presence of colloidal gold-ovumucoid complex at 25°C (see ref.). Freeze-fractured cells were also labeled with 1 mg/ml WGA-ferritin conjugates (E-Y lab, San Mateo, Ca.) in phosphate buffered saline for 1 hr. at 37°C. Salivary and pancreatic isolated cells were also fixed in 2% glutaraldehyde in cacodylate buffer, processed as above and, after fracture, thawing and deglycerination, treated for thiamine pyrophosphatase reaction, using the method of Novikoff and Goldfisher and labeled by WGA-colloidal gold as above. Freeze-fractured cells were incubated in solutions of Con A (0.25-0.5 mg/ml in 0.1M Sorensen phosphate buffer, 4% polyvinylpyrrolidone pH 7.4 for 1 hr at 25°C. Controls were preincubated in 0.4% methyl- $\alpha$ -D-mannopyranoside for 15 min at 37°C and then treated with Con A in presence of the sugar for 1 hr at 25°C.

**Processing for Electron Microscopy:** Fracture-labeled cells and tissue fragments were fixed in 1% osmium tetroxide in veronal acetate buffer pH 7.6 for 120 min. at 4°C, stained en bloc with uranyl acetate (5 mg/ml), dehydrated in acetone and embedded in Epon 812. Thin sections were obtained and examined unstained or post-stained with uranyl acetate and lead citrate.

Major Findings:

Recently, we showed that cytochemical and immunochemical techniques can be combined with freeze-fracture to gain generalized access for direct labeling of the fracture faces of biological membranes as well as of exposed groups in cross-fractured cytoplasm. The results of these "fracture-label" techniques can be assessed either by observation of thin sections of freeze-fractured specimens ("thin-section fracture-label") or of platinum/carbon replicas of critical point dried, freeze-fractured preparations ("critical-point-drying fracture-label").

Initial application of thin-section fracture-label showed that numerous concanavalin A binding sites could be labeled on the membrane faces of freeze-fractured plasma membranes, endoplasmic reticulum, and nuclear envelope membranes of leukocytes, HeLa cells, and hepatocytes. We report here the results of thin-section and critical-point-drying fracture-label of isolated rat exocrine and endocrine pancreatic cells. We used colloidal gold and ferritin conjugates to determine and compare the patterns of distribution of wheat germ agglutinin (WGA) and concanavalin A (Con A) binding sites on the fracture face of their plasma and intracellular membranes. Our results show that whereas Con A binding sites are present on nuclear envelope, endoplasmic reticulum, secretory vesicle, and plasma membranes, wheat germ agglutinin binding sites are absent from the nuclear envelope, the endoplasmic reticulum, mitochondria and peroxisome membranes, but strongly label primary and secondary lysosome membranes as well the plasma membrane. Over fractured golgi membranes distribution of Con A and WGA binding sites appears complex and must be analyzed in conjunction with the known functions of this membrane system as a center for glycosylation and sorting of membrane glycoproteins. These results are consistent with current views on the pathways of glycosylation of membrane proteins and do not support the reflux of fully glycosylated products to the endoplasmic reticulum. In addition, they demonstrate the capacity of fracture-label to investigate the topochemistry of plasma and intracellular membranes in situ.

Significance to Biomedical Research and the Program of the Institute:

Study of the mechanisms of glycosylation of membrane proteins is at present pursued in many laboratories, generally involving biochemical approaches. Because cell fractionation is necessary, cross contamination of membrane fractions makes it difficult to ascribe precise locations to glycosylated products. Cytochemical investigations rely on and labelling of autoradiography (with limited resolution) and frozen thin sections, an approach of unusual difficulty and limited scope. Our "fracture-label" techniques circumvent these problems and permit -- in an easy an straightforward manner -- not only to locate a variety of membrane components but also to learn about their pattern of distribution and differential association with each membrane half. Our study provided new insights about the intra localization of membrane glycoproteins. Having now learned about the distribution and topology of these glycoproteins in the membranes of normal cells, we are now in a favorable position to study any departures that may characterize pathological conditions, particularly those characteristics of malignant transformation.

Proposed Course of Research:

Goals of the project have been achieved. This research approach will be continued in the study of labelling patterns in normal and transformed cells as well as in healthy tissues, malignant tumours, and hormone-dependent regressing tumours.

Publications:

Pinto da Silva, P., Torrisi, M.R., and Kachar, B.: Freeze-fracture cytochemistry: Localization of wheat germ agglutinin and concanavalin A binding sites on freeze-fractured pancreatic cells. J. Cell Biol. 91: 361-372, 1981.

Torrisi, M.R. and Pinto da Silva, P.: Compartmentalization of sialoglycoconjugates in intracellular membranes: a fracture-label study. Proc. Natl. Acad. Sci., submitted for publication.

Pinto da Silva, P.: Freeze-fracture cytochemistry: Labelling of plasma and intracellular membranes. Cienc. Biol. (Cell Mol. Biol.), in press.

Torrisi, M.R. and Pinto da Silva, P.: Fracture label of golgi and lysosomal membranes. Abstract submitted to the 22nd Annual Meeting of the American Society of Cell Biology, 1982.

Pinto da Silva, P., Torrisi, M.R. and Kachar, B.: Localization of WGA and Con A binding sites on freeze-fractured pancreatic cells. J. Cell Biol. 91: 262a, 1981.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08271-01 LPP												
PERIOD COVERED October 1, 1981 to September 30, 1982														
TITLE OF PROJECT (80 characters or less)  T Lymphocyte Heterogeneity: Labeling of Lectin Receptors of Transmembrane Proteins														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 40%;">P. Pinto da Silva, Ph.D.</td> <td style="width: 25%;">Chief, Membrane Biology</td> <td style="width: 20%;">LPP, NCI</td> </tr> <tr> <td>Others:</td> <td>M. R. Torrissi</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td></td> <td>C. Parkison</td> <td>Chemist</td> <td>LPP, NCI</td> </tr> </table>			PI:	P. Pinto da Silva, Ph.D.	Chief, Membrane Biology	LPP, NCI	Others:	M. R. Torrissi	Visiting Fellow	LPP, NCI		C. Parkison	Chemist	LPP, NCI
PI:	P. Pinto da Silva, Ph.D.	Chief, Membrane Biology	LPP, NCI											
Others:	M. R. Torrissi	Visiting Fellow	LPP, NCI											
	C. Parkison	Chemist	LPP, NCI											
COOPERATING UNITS (if any) Dr. M.R. Torrissi, Institute of General Pathology, Faculty of Medicine, University of Rome														
LAB/BRANCH Laboratory of Pathophysiology														
SECTION Membrane Biology Section														
INSTITUTE AND LOCATION NIH, NCI Bethesda, Maryland 20205														
TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.6	OTHER: 0.2												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords)  We have described "fracture-label" techniques that permit direct cytochemical labeling of <u>freeze-fractured plasma</u> and <u>intracellular membranes</u> as well as <u>cross fractured cytoplasm</u> . We report here the use of "fracture-label" to investigate the <u>distribution</u> and <u>partition</u> of wheat germ agglutinin (WGA) receptor sites over the protoplasmic (P) and exoplasmic (E) plasma membrane faces of freeze-fractured human thymus derive (T) lymphocytes. All exoplasmic faces are strongly labeled by WGA. In contrast, the protoplasmic faces exhibit remarkable variation, ranging from virtual absence of label in some faces to very high densities in other faces. We interpret the presence of WGA receptor sites over the protoplasmic faces to reflect the presence of transmembrane, WGA binding sialoglycoproteins that, during freeze-fracture, partition with the inner half of the plasma membrane. Our results, therefore, indicate heterogeneous expression of integral membrane proteins within populations of human T cells. Fracture-label techniques thus represent an additional tool in the definition of lymphocyte subpopulations.														



## Methods:

For thin section fracture-label, human peripheral lymphocytes were isolated from fresh blood by Ficoll-Hypaque gradients and separated from adherent cells by incubation at 37°C for 30 min in plastic Petri dishes. The nonadherent cells were incubated in RPMI 1640 culture medium (37°C, 30 min, to remove Fc receptor bound Ig) and fixed in .5% glutaraldehyde (4°C, 30 min) to avoid antibody mediated capping in subsequent steps. Before embedding in the gel matrix the cells were treated with an IgG fraction of goat anti-human Ig (5 mg/ml, 25°C, 30 min) followed by protein A coated colloidal gold (25°C, 3 hrs). This procedure labeled the cell surface of B lymphocytes and Fc receptor-positive T cells and permitted their subsequent identification in thin sections. Thus, any cell that was labeled on the unfractured surface of the plasma membrane could be excluded from the analysis of fracture surfaces. The cells were then embedded in 25% bovine serum albumin (BSA), crosslinked by 1% glutaraldehyde and the resulting gels sliced, impregnated in 30% glycerol and frozen in Freon 22 cooled by liquid nitrogen. Frozen gels were fractured in liquid nitrogen by crushing with a glass pestle as described. Gel fragments were thawed, deglycerinated, washed, incubated with WGA (0.25 mg/ml; 37°C; 1 hr in 0.1M Sorensen's phosphate buffer with 4% polyvinylpyrrolidone pH 7.4) and labeled with ovomucoid coated colloidal gold (25°C, 3 hrs). Controls were pre-incubated in .4M N acetyl-D-glucosamine (Glc Nac) (37°C, 15 min), treated with WGA (0.25 mg/ml) in presence of the competing sugar and labeled with ovomucoid coated colloidal gold as above. The gel fragments were then dehydrated, embedded in Epon 812 and thin sectioned. For critical point dried fracture-label we used purified human T cells obtained by nylon wool filtration of Ficoll-Hypaque isolated lymphocytes. These cells were greater than 95% surface Ig negative as judged by staining with fluorescein isothiocyanate-labeled anti-human IgG. The T cell suspensions were fixed in glutaraldehyde, embedded in BSA, and the resulting gels sliced, glycerinated and frozen as above. Frozen gels were transferred to a Petri dish filled with liquid nitrogen and fractured with a pre-cooled scalpel. The fractured gels were thawed, deglycerinated, treated with WGA and labeled by ovomucoid coated colloidal gold as described above. Controls were labeled in the presence of Glc Nac as described. Labeled fragments were then dehydrated in acetone, critical point dried and replicated by Pt-C evaporation. The gels were digested in sodium hypochlorite (5%) and the replicas were cleaned, washed in water and observed by transmission electron microscopy.

## Major Findings:

Observation of the distribution of WGA receptor sites over fractured plasma membranes shows that while all exoplasmic halves are strongly labelled, labelling of protoplasmic faces presents remarkable heterogeneity: some cells are not labelled, others are sparsely labelled, whereas in a fraction (about 30%) the label is very dense. Because we could show that in pre-labelled cells WGA binding sites were absent from protoplasmic faces we conclude that WGA binding sites over these membrane halves represent the sites of integral transmembrane sialoglycoproteins. During fracture these proteins and their lectin receptors are pulled across the outer half of the plasma membrane. Therefore, the plasma membranes of human T cells contain a heterogeneous complement of transmembrane sialoglycoproteins. These molecules are unrelated to those containing bearing Con A binding sites (including putative molecules responsible for mitogen signaling).

Significance to Biomedical Research: Fracture-label appears to open a new approach to search and characterize lymphocyte subpopulations based on differences in the expression of transmembrane proteins. Heterogeneity of T cell populations, as revealed by fracture-label, retains possible operational value and a significance that is reinforced by the homogeneous labelling patterns so far observed in other cells as well as by the recent finding of multiple WGA binding glycoproteins in the plasma membranes of human T cells. The effects of stimulatory and inhibitory concentrations of WGA, the possible correlation with existent lymphocyte subpopulations (e.g. helper/suppressor), as well as the screening of fracture-labelled lymphocytes from patients with various types of T cell leukemias are, therefore, of immediate concern. In addition, we want to use monoclonal antibody directed against specific sites of integral transmembrane proteins (HLA, IA) to investigate the distribution and possible heterogeneous expression of transplantation antigens. We will also re-investigate lectin induced capping phenomena in order to find whether heterogeneity observed by fracture-label is also manifested leading to the possibility of routine laboratory screening of samples.

Proposed Course of Research: 1) Screening of fracture-labelled lymphocytes from patients with various types of T cell leukemias; 2) Screening of T cell subpopulations (helper/suppressor; use of monoclonal antibodies that recognized membrane antigens, e.g. OKT 4, OKT5 and OKT 8); 3) Search for labelling patterns in human B cells and monocytes; 4) Investigation of the distribution and partition of human and guinea pig transplantation antigens using monoclonal antibodies (provided by Dr. Anton Shevach).

#### Publications:

Torrisi, M.R. and Pinto da Silva, P.: T lymphocyte heterogeneity: wheat germ agglutinin labeling of transmembrane glycoproteins. Proc. Natl. Acad. Sci., in press.

Pinto da Silva, P. and Torrisi, M.R.: Subpopulations of T lymphocytes as revealed by fracture-label. In Fox, C.F. (Ed.): J. Cell. Biochem. Suppl. 6. Alan R. Liss, Inc., N.Y. Abstract submitted to UCLA Symposia Conference on Evolution of Hormone Receptor Systems, 1982.

Torrisi, M.R. and Pinto da Silva, P. Heterogeneity of expression of integral membrane proteins in human T lymphocytes. Abstract submitted to the 22nd Annual Meeting of the American Society for Cell Biology.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08272-01 LPP									
PERIOD COVERED October 1, 1981 to September 30, 1982											
TITLE OF PROJECT (80 characters or less)  Membrane glycoproteins and glycolipids of normal and transformed human cells											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT											
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: P. Pinto da Silva</td> <td style="width: 33%;">Membrane Biology Section</td> <td style="width: 33%;">LPP, NCI</td> </tr> <tr> <td>Others: Maria Luiza F. Barbosa,</td> <td>Visiting Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td>C. Parkison</td> <td>Chemist</td> <td>LPP, NCI</td> </tr> </table>			PI: P. Pinto da Silva	Membrane Biology Section	LPP, NCI	Others: Maria Luiza F. Barbosa,	Visiting Fellow	LPP, NCI	C. Parkison	Chemist	LPP, NCI
PI: P. Pinto da Silva	Membrane Biology Section	LPP, NCI									
Others: Maria Luiza F. Barbosa,	Visiting Fellow	LPP, NCI									
C. Parkison	Chemist	LPP, NCI									
COOPERATING UNITS (if any)											
LAB/BRANCH Laboratory of Pathophysiology											
SECTION Membrane Biology Section											
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.2	OTHER: 0.1									
CHECK APPROPRIATE BOX(ES)											
<input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER											
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords) <u>Glycoproteins</u> and <u>glycolipids</u> have been implicated in cell-cell interactions, cellular growth regulation and differentiation, as well as tumour antigens. Currently, we are investigating the distribution and partition of glycoproteins and glycolipids in the plasma and intracellular membranes of normal and SV40 virus-transformed WF-38 fibroblasts. The lectins concanavalin A(Con A), <u>Ricinus communis</u> agglutinin (RCA), Wheat Germ Agglutinin (WGA) and <u>Ulex europeus</u> agglutinin (UEA) are used as probes. It is our intention to characterize glycoproteins and glycolipids based on a) their partition behaviour during freeze-fracture; b) their resistance or susceptibility to selected enzymatic degradations and c) their presence after treatment of cell cultures with specific inhibitors such as tunicamycin.											

Methods:

The human diploid cell line WI-38 was obtained from the American Type culture collection and has been cultured in Basal medium (Eagle-diploid) supplemented with 10% fetal calf serum, 2% glutamine and antibiotics. Cultures of the SV40 virus-transformed derivative of the WI-38 cell line were acquired from Flow Laboratories.

Cells in the subconfluent stage are fixed *in situ* with 2% glutaraldehyde in PBS or culture medium scrapped with a rubber policeman and processed for fracture-label.

The fracture-label technique exposes the plasma and intracellular membranes to cytochemical label and reveals, at high resolution (better than 20 nm), the distribution and partition of components in freeze-fractured membrane halves.

Detection of Con A binding sites is done by label with colloidal gold coated with horseradish peroxidase. Label of WGA binding sites is obtained with colloidal gold coated with ovomucoid. RCA and UEA binding sites are detected by the use of lectin-ferritin conjugates.

Major findings:

The project was recently initiated. In a first attempt to analyze the distribution of lectin binding sites on the membranes of normal and transformed cells, we noticed a definitive decrease in the amount of binding sites present on P faces (protoplasmic face) of membranes from transformed cells. We believe the label found on P faces corresponds to transmembrane glycoproteins. It follows that, as previously proposed, transformed cells present a reduced amount of membrane glycoproteins when compared to normal cells. A detailed analysis of the distribution and partition of lectin binding sites in intracellular membranes is in process.

Significance to Biomedical Research: Glycoproteins and glycolipids have been implicated in cell-cell interactions, functions, cellular growth regulation and differentiation and as tumor antigens. It has been clearly shown that changes in glycoproteins and glycolipids patterns do occur during cell growth, differentiation and transformation. We are coupling the benefits of the tissue culture technique with the expedience of the fracture-label method to study the qualitative and quantitative alterations on glycoproteins and glycolipids patterns resultant from viral transformation.

Proposed Course of Research: The project is being developed by: a) analysis of the distribution of lectin binding sites among cellular membranes; b) analysis of the partition of lectin binding sites after freeze-fracture; c) study of the susceptibility of lectin binding sites to selected enzymatic degradations; d) localization of lectin binding sites after treatment of cell cultures with specific inhibitors such as tunicamycin.

Publications: work in progress.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08273-01 LPP												
PERIOD COVERED October 1, 1981 to September 30, 1982														
TITLE OF PROJECT (80 characters or less) The Biochemistry of Invasiveness														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT														
<table style="width: 100%; border: none;"> <tr> <td style="width: 40%;">PI: S. L. Berger</td> <td style="width: 40%;">Research Chemist</td> <td style="width: 20%;">LPP, NCI</td> </tr> <tr> <td>Others: W. H. Eschenfeldt</td> <td>Staff Fellow</td> <td>LPP, NCI</td> </tr> <tr> <td>L. A. Liotta</td> <td>Senior Surgeon</td> <td>LP, NCI</td> </tr> <tr> <td>U.P. Thorgeirsson</td> <td>Visiting Scientist</td> <td>LP, NCI</td> </tr> </table>			PI: S. L. Berger	Research Chemist	LPP, NCI	Others: W. H. Eschenfeldt	Staff Fellow	LPP, NCI	L. A. Liotta	Senior Surgeon	LP, NCI	U.P. Thorgeirsson	Visiting Scientist	LP, NCI
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COOPERATING UNITS (if any) Laboratory of Pathology														
LAB/BRANCH Laboratory of Pathophysiology														
SECTION Office of the Chief														
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205														
TOTAL MANYEARS: 2.0	PROFESSIONAL: 2.0	OTHER: 0												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords) The effect of interferons on the <u>invasiveness in vitro</u> of human malignant cells derived from Ewing's sarcoma was evaluated. When treated with either crude or homogeneous fibroblast or lymphoblastoid interferon for 6 days, the cells responded by producing <u>type IV collagenase</u> at levels 2- to 10-fold higher than control values. The ability of Ewing's sarcoma cells to invade <u>human amnion connective tissue</u> was also determined. After 6 days of exposure to <u>crude leucocyte</u> , <u>homogeneous lymphoblastoid</u> or <u>homogeneous fibroblast interferon</u> , invasiveness of the treated samples increased 3-, 17- and 22-fold, respectively, relative to that of untreated controls.														



Although the antiproliferative activity of interferons both in vitro and in vivo is well documented in the human system, the direct effect of these substances on metastatic behavior has not been sufficiently explored. Previous studies of the effect of interferon on metastasis in vivo could not distinguish among effects on tumor cell intrinsic invasive behavior, on tumor cell proliferation and on host immune responses. The problem of studying invasive behavior, exclusively, has been approached by evaluating two parameters in vitro: the amount of type IV (basement membrane) collagenase secreted by cells in culture; the capacity of cells to penetrate human amnion basement membrane and collagenous stroma. Since the ability of a cell to degrade type IV collagen in vitro correlates with the incidence of spontaneous lung metastases derived from a transplanted tumor of the same cell type in vivo, these studies measure phenomena crucial to one aspect of the complex metastatic process.

Cultured Ewing's sarcoma cells grown by standard tissue culture methods were used throughout the study. When they were administered subcutaneously to the subscapular region of 3-week old athymic nude mice, tumors developed that, on microscopic examination after 6 weeks, demonstrated features typical of Ewing's sarcoma. The cultured cells, therefore, retained their malignant phenotype.

Ewing's cells were also capable of developing the antiviral state in response to interferon. When challenged with encephalomyocarditis virus after exposure to 100 Units/ml of various crude interferons, the interferon treated cells, survived whereas controls did not. The sensitivity of Ewing's cells to interferons was roughly equivalent to human foreskin fibroblasts, a cell type commonly used for interferon assays.

The amount of type IV collagenase secreted by Ewing's cells in serum-free medium in the presence and absence of various types of interferon was followed for 6 days. When incubated with crude lymphoblastoid or fibroblast interferon at either 10 or 100 Units/ml, measurements of the rate of appearance of type IV collagenase in the cell supernatant fluids revealed no dramatic changes relative to controls throughout 4 days of treatment. When similar measurements were made after 6 days of exposure, the rate of collagenase production per cell increased 2- to 4- fold relative to control levels. However, both these crude interferons were toxic to the cells, hence no absolute increases in collagenase levels occurred. In contrast, crude leukocyte interferon which was nontoxic stimulated type IV collagenase production approximately 2-fold relative to control values. These results suggested that impurities in some interferon preparations, not the interferons themselves, were the cause of cell death.

To avoid the toxicity of the crude materials two interferons purified to homogeneity, lymphoblastoid and fibroblast interferon, were tested. Both interferons elicited a small but reproducible antiproliferative effect after 6 days. In contrast, increases in type IV collagenase levels were discernible after only 2 days of treatment of the Ewing's cells with the purified materials and by 6 days of interferon-treatment the elevation in type IV collagenase titers relative to control values sometimes approached 10-fold.

The effect of interferons on type IV collagenase production by Ewing's cells was reflected by measurements of invasiveness. Invasive capacity was evaluated by

determining the number of cells able to traverse human amnion basement membrane stripped of the epithelial cell layer. The method was developed by Liotta and colleagues. Cells treated with 100 Units/ml crude leukocyte, homogeneous lymphoblastoid or homogeneous fibroblast interferon for 6 days in serum-free medium were washed free of interferons and allowed 2 or 4 days in which to invade the amnion. Invading cells could be captured on a filter lying immediately beneath the basement membrane. Relative to the behavior of Ewing's cells that had not been exposed to interferon, invasiveness of the treated cells increased 3-, 17- and 22-fold, respectively in the 4-day assay.

In a shortened version of the aforementioned assay, invasiveness of Ewing's cells was measured by exposing them to interferon and to the amnion simultaneously. Under these conditions, invasiveness of the treated cells relative to cells that had never been exposed to interferon increased 4- to 5-fold in two days when the purified materials were used. The stimulation of invasiveness in response to interferon in the abbreviated assay was much lower than that observed previously. However, the results are consistent with measurements of type IV collagenase levels in the cell culture fluids. Two days of interferon treatment are sufficient for neither the generation of maximum collagenase titers nor the induction of maximum invasiveness.

In interpreting these results it should be emphasized that the ability of interferon to stimulate type IV collagenase and to enhance invasiveness of Ewing's sarcoma cells in vitro cannot a priori be extended to other cell types or tumors in vivo. Any extrapolation of the data must take into consideration multiple effects on cell proliferation or intrinsic invasiveness, as well on the immune system of the host. Clearly, the relative importance of the various interactions of interferon with all aspects of the system, be it human or animal, must be weighed when evaluating this potent and many-faceted material.

Significance to Biomedical Research and the Program of the Institute: This project conforms to the Objective #3, Approach #1 of the National Cancer Plan. Its aims at an understanding of the biochemical control mechanisms by which normal cell growth and function are maintained. Disordered cell growth in neoplastic populations may then be better understood and rational attempts made to prevent or modify it.

The project also conforms to Objective #2, Approach #2. Since interferon produced in vivo or administered as an external agent may be effective in preventing or curing some types of cancer, it is essential to understand both its modes of production and its mechanism of action in normal, virus-treated and malignant cells.

Proposed Course of Research: Invasiveness of other cells in the presence and absence of interferons will be studied. Long term goals include understanding the various aspects of invasive capacity at the molecular level.

#### Publications:

Siegal, G.P., Thorgeirsson, U.P., Russo, R.G., Wallace, D.M., Liotta, L.A. and Berger, S. L. Interferon enhancement of the invasive capacity of Ewing's sarcoma cells in vitro. Proc. Natl. Acad. Sci., USA, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08274-01 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Regulation of Lactogenic Hormone Receptors in Mammary Tissue		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: B.K. Vonderhaar Others: T.M. Horn	Research Chemist Staff Fellow	LPP, NCI LPP, NCI
COOPERATING UNITS (if any)		
Laboratory of Pathophysiology		
Title of the Chief		
Name, Title and Address		
NHLBI, NIH, Bethesda, Maryland 20205		
TOTAL MAN-YEARS:	PROFESSIONAL:	OTHER:
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project is designed to evaluate the nature of <u>lactogenic hormone receptors</u> and the factors (including other hormones) which affect binding of the hormone to this molecule. Studies include 1) purification of the receptor and preparation and characterization of an antibody against it; 2) examination of the nature of the interaction of <u>prolactin</u> and <u>human growth hormone</u> with native as well as cryptic forms of the receptor; 3) characterize the selectivity of the effect of changes in the <u>membrane lipid</u> environment on binding of lactogenic hormones to their receptors vs other peptide hormones (such as <u>EGF</u> ) binding to their receptors, and 4) examining the metabolism of prolactin by its target tissues		

Description:

The purpose of these studies is to investigate the nature of the interaction of lactogenic hormones with their receptors, the stability of the hormone-receptor complex, and the nature of the cryptic sites as well as the effects of divalent cations, alterations in membrane lipids and membrane aggregation. The selectivity of these effects on lactogenic hormone receptors was determined by examining similar effects on somatogenic receptors or the receptors for EGF. Purification of the lactogenic hormone receptors was attempted in order to prepare antibodies for studies on regulation of synthesis of the molecule. Methods were developed to prepare internally labeled prolactin in order to study the fate of this hormone.

Methods Employed:

C3H/HeN mice were used. Hormones were iodinated by a lactoperoxidase method. Microsomal membranes were prepared from mammary glands (MG) or livers (L) of 10-12 day lactating or 10-12 day mid-pregnant mice using established procedures. Specific binding of prolactin (PRL), human growth hormones (hGH), or EGF to membrane bound or detergent solubilized receptors was studied by standard competition binding techniques. Solubilization of receptors was performed either in the presence of 1% Triton X-100 or 0.5-1.0% of the zwitterionic detergent CHAPS.

Nb2 rat lymphoma cells (obtained from Drs. Gout and Noble) are propagated in Fischer's leukemic cell medium with 0.15 mM  $\beta$ -mercaptoethanol, 10% horse serum and 10% fetal calf serum. Rat GH<sub>3</sub> cells (obtained from Dr. Bancroft) are propagated in Joklik's modified medium containing 15 mM HEPES, 12.5% horse serum and 2.5% fetal calf serum. Growth of cells is monitored by direct counting using a hemacytometer.

Major Findings:

In order to understand the regulation of PRL binding and induction of cryptic binding sites, we examined the role of membrane protein aggregation and fluidity changes induced by Concanavalin A (Con A) on subsequent hormone binding. The binding of hGH to lactogenic hormone receptors in the membrane bound vs the Triton solubilized form was examined in the presence of Con A. The binding to membranes from either mammary glands (MG) or livers (L) was inhibited by Con A to about 40-50% while hGH binding to the soluble form was unaffected. The Con A inhibition was not due to a general effect on membrane proteins as binding of bovine growth hormone (bGH) to the somatogenic binding sites on the membranes was unaffected. This difference in sensitivity of lactogenic vs somatogenic sites to Con A action is probably due to a lack of Con A binding sites associated with the somatogenic receptor. Solubilized lactogenic hormone receptors as well as solubilized pre-bound PRL-receptor or hGH-receptor complexes are retained on Con A-Sepharose columns. Triton solubilized bGH-receptor complexes are not retained by Con A-Sepharose. Once bound to receptors, lactogenic hormones are not released by the action of Con A. Inhibition of binding to lactogenic receptors by Con A occurs independently from other membrane perturbing events such as phospholipid methylation.



The selectivity of the effects of membrane methylation were examined in detail in both MG and L membranes. For these studies lactogenic hormone binding is defined as  $^{125}$ I-hGH displaced by unlabeled ovine prolactin (oPRL).  $^{125}$ I-mEGF is displaced by unlabelled mEGF. Membranes pre-treated with the methyl donor S-adenosyl-L-methionine (SAM) for 5 min at 37° in the absence of hormones showed maximum stimulation of specific binding of lactogenic hormones. Scatchard analysis showed as much as 4-fold increase in MG membrane receptor number in buffer A (25 mM Tris-HCl pH 8.0/10 mM  $MgCl_2$ /0.1% BSA) with a negligible effect on receptor affinity.

Under similar pre-incubation conditions, the effect of SAM on lactogenic hormone and EGF binding to MG and L membranes was examined in a series of buffers to control for ionic strength, pH and divalent cations. Besides buffer A listed above, the following buffers, were examined; B, 50 mM Tris-glycylglycine pH 8.0/10 mM  $MgCl_2$ /0.1% BSA; C, PBS pH 7.4/0.1% BSA; D, PBS pH 8.0/0.1% BSA; E, PBS pH 8.0/10 mM  $MgCl_2$ /0.1% BSA. All binding reactions were terminated and membrane pellets washed with 25 mM Tris-HCl pH 7.4/10 mM  $MgCl_2$ /0.1% BSA.

In buffers A, B, and C SAM greatly stimulated lactogenic hormone binding to lactating mouse MG (2-3.7 fold). Lactogenic hormone binding to L membranes was minimally affected in buffers A (20%) and B (12%) but in buffer C, a 2-fold increase was observed. In contrast, EGF binding to MG was slightly suppressed in buffers A and B, but slightly stimulated (<20%) in buffer C. EGF binding to L was unaffected by SAM in all 3 buffers.

These results suggested that  $Mg^{+2}$  might be important in mediating differential effects of SAM on lactogenic hormone binding to L and on EGF binding to MG. Therefore, buffers D and E were employed which differ only in  $Mg^{+2}$  content. In buffer D (no  $Mg^{+2}$ ) EGF binding to MG was increased by SAM treatment ( $1.48 \pm 0.18$  fold) while buffer ( $+Mg^{+2}$ ) EGF binding was suppressed ( $0.87 \pm .11$ )

EGF binding to microsomes from both L and MG of pregnant mice was also unresponsive to SAM treatment. Detailed Scatchard analysis showed that the slightly suppressed binding of EGF in the presence of  $Mg^{+2}$  results from loss of low affinity binding sites. In liver, the SAM induced increase in lactogenic hormone binding seen in buffer C appears to be due to an increase in the number of low affinity binding sites. Thus, the high affinity sites for lactogenic hormone binding in L as well as MG are unaffected by SAM. The high affinity sites for EGF in MG are sensitive to the presence of  $Mg^{+2}$ . Lactogenic hormone binding in MG is increased by SAM  $\pm Mg^{+2}$ . These differential sensitivities to  $Mg^{+2}$  may reflect the differences in ion requirements for the two methyltransferases involved in synthesis of phosphatidylcholine (PC) from phosphatidylethanolamine (PE). The first enzyme requires  $Mg^{+2}$  and has a low  $K_m$  for SAM; the second does not require  $Mg^{+2}$  and has a high  $K_m$  for SAM.

Preliminary work has begun in an attempt to correlate methylation and activation of lactogenic hormone receptors with the biological activity of the hormone and its metabolism by target cells. This has centered around production of PRL by rat GH<sub>3</sub> cells. Conditioned medium from these cells grown under serum-free



conditions with an without 0.4 mM  $\text{CaCl}_2$  was used as the source of prolactin. Aliquots of the media were added to the highly PRL-sensitive Nb2 cells in the presence of horse serum. Growth of the Nb2 cells was monitored after 3-4 days by counting cells in a hemacytometer. Initial findings show that GH<sub>3</sub> cells produce a potent Nb2 cell growth factor presumably rat prolactin which can be labeled with radioactive amino acids to follow its metabolism.

In order to study the regulation of the lactogenic hormone receptor itself, purification of the prolactin receptor from lactating mouse livers was attempted. Previous attempts utilized the non-ionic detergent Triton X-100 to initially solubilize the receptor from membranes. The resulting solubilized binding unit, however, was either aggregated with itself or with other membrane associated proteins. Therefore, purification of the receptor was not very successful.

Another detergent, CHAPS (3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate) a zwitterionic derivative of cholic acid was used. CHAPS at concentrations as high as 0.5% did not affect the molecular characteristics of  $^{125}\text{I}$ -oPRL, thus allowing this lactogenic hormone to be used in the binding assay. ( $^{125}\text{I}$ -oPRL aggregates in the presence of even 0.1% Triton X-100 and, therefore, could not previously be used with solubilized receptors). CHAPS at 0.5 to 1% solubilizes 45-65% of membrane proteins and lactogenic hormone (PRL) receptors.  $^{125}\text{I}$ -oPRL-receptor complexes are effectively separated from unbound  $^{125}\text{I}$ -oPRL by precipitation with polyethylene glycol (PEG; 12.5% w/v).

For maximal binding of  $^{125}\text{I}$ -oPRL to CHAPS solubilized receptors, the detergent should be present in the binding reaction at a final concentration of 0.4%. The divalent cation  $\text{Mg}^{+2}$  at 10 mM must be present in both the binding reaction and the PEG precipitation step. The CHAPS solubilized receptor retains the specificity for lactogenic hormones present in particulate membranes.

Scatchard analysis showed the same binding affinity ( $2-4 \times 10^9 \text{ M}^{-1}$ ) for oPRL for both soluble and particulate receptors. Solubilized  $^{125}\text{I}$ -oPRL-receptor complexes are stable (~85% of hormone remains bound) for at least 48 hr at room temperature even in the presence of excess unlabeled oPRL.

By gel filtration on a calibrated Sephadex G-100 column the molecular weight of the hormone receptor complex was found to be 57,000-61,000 daltons. This represents a complex of PRL with a single peptide of molecular weight of  $37,000 \pm 2,000$ .

Since the solubilization proces using CHAPS did not alter the affinity and specificity of the receptor and did not affect the stability of the PRL-receptor complex, we attempted "one-step" affinity purification of this receptor.

An affinity gel was prepared by coupling oPRL to CH-Sepharose 4B (with a hydrophobic 6 carbon spacer arm). The solubilized receptors were mixed with the affinity matrix for 8-10 hr at room temperature and then the non-specifically absorbed proteins extensively eluted with solutions of 25 mM Tris-HCl pH 7.4/0.5% CHAPS/1% Lubrol-P and 25 mM Tris-HCl pH 7.4/0.5% CHAPS/0.5 M NaCl. The receptors were then eluted with 4M urea/1M NaCl/0.5% CHAPS followed by 5M  $\text{MgCl}_2$ /0.5% CHAPS. After extensive dialysis the active fractions from the column were concentrated

and analyzed. The eluted receptor appeared on SDS-PAGE as a single major band with a molecular weight of 37,000. When passed through a calibrated Sephadex G-100 column, the PRL receptor complex had an apparent molecular weight of 57,000-61,000 confirming the 37,000 dalton band as the binding protein.

The purified receptor retains its specificity for lactogenic hormones and binds  $^{125}\text{I}$ -OPRL with a  $K_d$  of  $2$  to  $6 \times 10^{-9} \text{ M}^{-1}$  as does the particulate form.

This purified receptor has been injected into rabbits to raise an antibody. Preliminary characterization of the antiserum has been by the Ouchterlony double diffusion method. The anti-serum but not pre-immune serum, reacts against the purified antigen and a component of mouse whey (possible receptors from milk fat globule membranes known to carry PRL receptors). The antiserum does not react against purified mouse caseins, mouse  $\alpha$ -lactalbumin, disrupted MMTV from mouse mammary tumors and whole mouse serum from tumor bearing mice.

Significance to Biomedical Research and the Program of the Institute: Prolactin-thyroid interactions are important in growth and differentiation of mammary glands. Altered thyroid status may be implicated in changes in risk of human breast cancer. Our studies are aimed at understanding whether changes in thyroid status play a direct or indirect role in mammary tumorigenesis. Thyroid hormones are known to regulate prolactin action through control of lactogenic receptors. Therefore, all aspects of prolactin binding and control of exposed as well as cryptic sites must be examined. Preparation of antibodies against the lactogenic receptors will readily allow us to study the receptor molecule itself in the absence of the hormone and thus determine if lack of prolactin binding in certain mammary tumors is due to lack of receptors or only their existence in masked forms. Changes in membrane phospholipids can greatly affect the capacity of a target cell to recognize and subsequently respond to specific hormones and drugs. Certain chemotherapeutic agents and drugs such as deazaadenosine, 5-azacytidine and methotrexate interfere with cellular methylation. How these agents affect membrane methylation and hormonal responsiveness of normal vs tumor tissue is of great interest.

Proposed Course of Research: We will continue to study control of lactogenic receptors by thyroid hormones. Work will involve studies on membrane composition and fluidity. Phospholipid methylation in various thyroid states and in the presence of  $\text{T}_3$  in culture will be examined. The effects of other hormones, which act on the mammary gland, as well as certain chemotherapeutic drugs will be examined in terms of membrane phospholipid methylation. The nature of the products formed in the presence and absence of  $\text{Mg}^{+2}$  will be determined including the possibility that specific membrane proteins are also methylated. The effects of membrane methylation on lactogenic hormone and EGF binding to mammary tumor membranes will be examined and compared to the hosts' normal mammary tissue. The effects of methylation inhibitors on hormone metabolism and specific protein synthesis in mammary explants and growth of the Nb2 rat lymphoma cell line will be examined. Using the antibody against PRL receptors we will examine the regulation of receptor synthesis and availability of this molecule to the hormone. We will purify the receptor from other tissue sources (especially mammary glands) and other species (rat, rabbit and human). A monoclonal antibody against the human prolactin receptor will be prepared.

Publications:

Bhattacharya, A. and Vonderhaar, B.K.: Membrane modification differentially affects the binding of the two lactogenic hormones--human growth hormone and ovine prolactin. Proc. Natl. Acad. Sci. USA 78: 5704-5707, 1981.

Alhadi, T. and Vonderhaar, B.K.: Induction of cryptic lactogenic hormone binding in livers of adult female mice treated neonatally with estradiol or nafoxidine. Endocrinology 110: 254-259, 1982.

Bhattacharya, A. and Vonderhaar, B.K.: Interaction of concanavalin A with lactogenic receptors in isolated membrane. Arch. Biochem. Biophys. 215: 425-432, 1982.

Liscia, D.S., Alhadi, T. and Vonderhaar, B.K.: Solubilization of active prolactin receptors by a non-denaturing zwitterionic detergent. J. Biol. Chem. 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08275-01 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Asymmetric topology of glycolipids in membranes of <u>Acanthamoeba castellanii</u>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: P. Pinto da Silva Other M. Luiza F. Barbosa C. Parkison	Chief, Membrane Biology Visiting Fellow Chemist	LPP, NCI LPP, NCI LPP, NCI
COOPERATING UNITS (if any)  Dr. Blair Bowers, NHLBI		
LAB/BRANCH Laboratory of Pathophysiology SECTION Membrane Biology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 1.1	PROFESSIONAL: 1.0	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (X) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>           We analyzed the <u>partition and distribution</u> of a <u>glycolipid</u> on the fracture faces and membrane surfaces of <u>Acanthamoeba castellanii</u>. The glycolipid, a <u>lipophosphoglycan</u> (LPG) with Concanavalin A (Con A) receptor sites, was labeled with colloidal gold coated with horseradish peroxidase. The label was abundant over exoplasmic faces but absent from protoplasmic faces. Access of lectin and colloidal gold to the interior of lysed cells confirmed the existence of Con A binding sites on the exoplasmic surfaces of vacuoles, but cytoplasmic surface of all cell membranes remained virtually unlabelled. Our results demonstrate the <u>asymmetric topology</u> of <u>glycolipid</u> molecules in a biological membrane. <u>Fracture-label</u> is proposed as a new technique to investigate the distribution and partition of glycolipids in plasma and intracellular membrane halves.         </p>		

Methods:

Acanthamoeba castellanii cultured axenically was generously provided by Dr. B. Bowers. Cultures in logarithmic growth phase were used in all experiments. Cells were fixed at 4°C for 30 min in 3% glutaraldehyde solution in 0.1 M sodium phosphate buffer pH 6.8. Fixed cells were processed for fracture-label.

The fracture-label technique exposes the plasma and intracellular membrane to cytochemical label and reveals, at high resolution (better than 20 nm), the distribution and partition of components in freeze-fractured membrane halves. Fracture-label bypasses the problems inherent to techniques of cell disruption and membrane fractionation.

Detection of Con A binding sites was done by label with colloidal gold coated with horseradish peroxidase.

Major Findings:

Our results were the first cytochemical demonstration of the asymmetric topology of glycolipid molecules in a biological membrane. We propose fracture-label as a new technique to investigate the distribution and partition of glycolipids in plasma and intracellular membrane halves.

Significance to Biomedical Research: Glycolipids are important components of biological membranes. Among many functions, they serve as receptors, antigen determinants or as regulators of cell behavior. Investigation of the topology of glycolipids in biological membranes is difficult due to the cryptical disposition of some glycosphingolipids. In this work, we propose fracture-label as a new technique to investigate the distribution and partition of glycolipids in plasma and intracellular membrane.

Proposed Course of Research: All experimental objectives were realized with success. No new experiments necessary.

Publications:

Barbosa, M.L.F. and Pinto da Silva, P. Asymmetric topology of glycolipids in membranes: Concanavalin A labeling of membrane halves in Acanthamoeba castellanii. Cell, submitted for publication.

Barbosa, M.L.F. and Pinto da Silva, P. Application fracture-label cytochemistry in the localization of membrane glycolipids. J. Cell Biol., abstract submitted.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER 201 CB 08276-01 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Role of Calcium in Mediating Cell Growth and Differentiation		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: W. B. Anderson                      Research Chemist                      LPP, NCI  Others: R. Gopalakrishna              Visiting Scientist                      LPP, NCI A. Kraft                          Research Associate                      LPP, NCI		
COOPERATING UNITS (if any)		
LABORATORY Laboratory of Pathophysiology		
SECTION Office of the Chief		
NOT, TIME, AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL YEARS: 2.0	PROFESSIONAL:      2.0	OTHER:                  0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Characterization studies have been carried out on a protein kinase which is stimulated by both calcium and phospholipids. Differentiated <u>PYS endoderm cells</u> possess <u>calcium, phospholipid - dependent protein kinase (Ca, PL-PK)</u> activity while no such activity could be detected in undifferentiated F9 cells. Retinoic acid- induced differentiation of F9 cells to an endoderm cell type provoked a time-dependent increase in cytosolic Ca, PL-PK activity. This increase in Ca, PL-PK activity correlates with differentiation to the parietal endoderm cell type. Employing <u>EL 4 thymoma cells</u> as a source of enzyme, it was found that cytosolic Ca, PL-PK activity is markedly decreased following treatment of the cells with <u>phorbol ester</u> . The decrease occurs within 5 min after the addition of phorbol ester and is dose dependent. Alteration of this protein kinase activity conceivably mediates later effects of the phorbol ester tumor promoter. It was found that the calcium binding protein, calmodulin, contains a calcium-induced hydrophobic site. Using hydrophobic interaction chromatography a single step procedure was developed to rapidly purify calmodulin to homogeneity.		

Project Description:

Objectives:

To identify biological activities controlled by calcium which might serve to regulate early biochemical events involved in mediating embryonic growth and differentiation.

Methods Employed:

Cell culture, standard biochemical analysis of calmodulin and protein kinase activities, hydrophobic interaction chromatography, affinity chromatography, SDS-polyacrylamide gel electrophoresis and radiographic analysis.

Major Findings:

The adenylate cyclase systems of F9 and PYS cells are stimulated by calcitonin and parathyroid hormone, respectively. Since these two hormones also are known to modulate calcium flux it is of interest to identify activities present in these cells which might be modulated by changes in calcium levels. Initial studies have dealt with the characterization of the calcium binding protein, calmodulin. Taking advantage of the finding that calcium binding to calmodulin induced a hydrophobic site on this protein, a one step purification was devised utilizing hydrophobic interaction chromatography. This purification procedure offers definite advantages over previously published methods, and will greatly facilitate the study of this important regulatory protein.

Other studies have dealt with the characterization of a calcium, phospholipid-dependent protein kinase (Ca, PL-PK) activity. Initially, Ca, PL-PK activity could not be detected in supernatants prepared from either F9 or PYS cells. However, passage of PYS cytosol over a DEAE-cellulose column revealed Ca, PL-PK activity which eluted with 0.045 M NaCl. The partially purified PYS enzyme has a Mr ~ 66 K and exhibits an app Ka for  $\text{Ca}^{2+}$  of 32  $\mu\text{M}$ . This enzyme is stimulated by acidic phospholipids, and diacylglycerol markedly increases histone H1 phosphorylation in the presence of  $\text{Ca}^{2+}$  and phospholipids. No Ca, PL-PK activity was found in undifferentiated F9 cells even when the cytosol was passed over a DEAE-cellulose column. Treatment of F9 cells with retinoic acid to induce differentiation to an endoderm cell type provokes a time-dependent increase in cytosolic Ca, PL-PK activity. These findings indicate that cytosolic Ca, PL-PK activity is very low, or nonexistent, in undifferentiated stem cells. With differentiation to a parietal endoderm cell type there is a marked increase in soluble Ca, PL-PK activity.

Since phorbol esters rapidly affect phospholipid metabolism and modulate calcium fluxes in many cells, we examined the effect of phorbol esters on Ca, PL-PK activity. EL 4 mouse thymoma cells, which were found to have high Ca, PL-PK activity, were utilized as the source of enzyme. Phorbol esters (3-100 nM) had no effect on Ca, PL-PK activity when added directly to the assay. Preincubation of the cells with phorbol esters, however, markedly decreased the activity of the kinase in cytosol preparations. The loss in activity was maximal (approximately 90% decrease) within 5 min at 37°C. Half maximal inhibition was achieved

at 4-5 nM phorbol 12-myristate, 13-acetate. The potency of a series of phorbol esters in decreasing Ca, PL-PK activity correlated with competition for binding to the specific phorbol ester receptor. These results suggest that the phorbol ester-mediated decrease in Ca, PL-PK activity may be regulated by the specific phorbol ester receptor. Alteration of this kinase activity may be important in modulating later phorbol ester effects.

Significance to Biomedical Research and the Program of the Institute:

Embryonal carcinoma cells are a malignant cell type which is developmentally analogous to the early post-implantation mouse embryo, and thus offers an excellent system to study the biochemical events involved in embryonic differentiation and development. It has been suggested that malignant stem cells show continued proliferation because neoplastic conversion has negated the ability of normal signals to induce differentiation. Thus, it is of importance to understand the mechanism by which retinoic acid acts to serve as a signal for the differentiation of malignant stem cells to non-malignant endoderm cells, or to understand the mechanism by which phorbol esters antagonize retinoic acid effects and promote the growth of tumors. Since calcium has been implicated as a modulator of cell growth and differentiation, it is of interest to establish which activities under calcium regulation might play a role in mediating the differentiation process.

Proposed Course of Research: Studies will continue on the characterization of activities present in embryonal carcinoma cells which might be modulated by changes in calcium levels, and to determine the role of calcium-dependent activities in the regulation of cell growth and differentiation. This will include efforts to prepare a specific antibody for immunofluorescent localization studies of calmodulin, and to establish a radioimmunoassay for quantitation of calmodulin. With these reagents studies can be carried out to determine if treatment of F9 cells with hormones such as somatomedins or calcitonin alters the intracellular levels or localization of calmodulin. Investigations also will deal with the nature of calmodulin interaction with target proteins, and will attempt to establish which activities under calmodulin control might respond to rapid changes in calcium to mediate growth and differentiation.

Other studies will be concerned with elucidating the mechanism by which phorbol esters dramatically decrease calcium phospholipid-dependent protein kinase activity, and with determining if this change in kinase activity is required to mediate other effects of the tumor promoter. An attempt will be made to purify the enzyme from rat tissue. If sufficient quantities can be obtained, antisera or monoclonal antibodies can be prepared to use in localization studies, or to detect active and inactive forms of the enzyme, to follow any changes which might occur during the rapid inactivation induced by phorbol ester tumor promoter.

Publications:

Gopalakrishna, R. and Anderson, W.B.:  $\text{Ca}^{2+}$ -induced hydrophobic site on calmodulin: application for purification of calmodulin by phenyl-sepharose affinity chromatography. Biochem. Biophys. Res. Commun. 104: 830-836 (1982).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 CB 08277-01 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Antibodies as probes of cyclic nucleotide function in human breast cancer cells		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <div style="width: 30%;">           PI: C. L. Kapoor            Other: Y.S. Cho-Chung         </div> <div style="width: 40%; text-align: center;">           Visiting Scientist            Chief, Cellular Biochemistry Sec. LPP, NCI         </div> <div style="width: 30%; text-align: right;">           LPP, NCI            LPP, NCI         </div> </div>		
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Cellular Biochemistry Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: <div style="border: 1px solid black; width: 50px; text-align: center; margin: 0 auto;">0</div>	PROFESSIONAL: <div style="border: 1px solid black; width: 50px; text-align: center; margin: 0 auto;">1.3</div>	OTHER: <div style="border: 1px solid black; width: 50px; text-align: center; margin: 0 auto;">0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div style="width: 30%;"> <input type="checkbox"/> (c) NEITHER  <div style="text-align: center;">X</div> </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)  <p> <u>Antibodies against bovine type I and type II cAMP-dependent protein kinase regulatory subunits R<sup>I</sup> and R<sup>II</sup></u>, respectively, were raised in rabbits and were purified by utilizing a new <u>cross-linked affinity chromatography</u> procedure. These antibodies specifically cross-reacted with the <u>human breast cancer cells (MCF-7 and MDAMB-231)</u> as determined by <u>radioimmunoassay</u> and <u>immunoprecipitation</u>. The <u>immunocytochemical</u> studies utilizing these antibodies revealed that R<sup>II</sup>, but not R<sup>I</sup>, is specifically compartmentalized in the <u>nucleoli</u> and <u>mitotic spindles</u> of these cancer cells, suggesting that R<sup>I</sup> and R<sup>II</sup> are involved in the different nuclear directed events in cancer cells. The photo-affinity labelling of the R proteins of MCF-7 cells revealed the presence of 50 K isoprotein of type II cAMP-dependent kinase. The physiological function of the 50 K protein in relation to its <u>subcellular compartmentalization</u> during different phases of <u>growth</u> is currently under investigation.         </p>		



Objectives:

- 1) To produce specific affinity purified antibodies against regulatory subunit of type I and type II cAMP-dependent protein kinase which could be effectively utilized to determine the subcellular compartmentalization of cAMP-dependent protein kinases in human breast cancer cells.
- 2) To identify the cyclic AMP-receptor proteins in estrogen receptor positive and negative human breast cancer cells.
- 3) To explore the regulation and function of cAMP-dependent protein kinases in breast cancer cells during different phases of growth in vitro and in vivo.

Methods Employed:

- 1) Bovine skeletal muscle R<sup>I</sup> and bovine heart R<sup>II</sup> cAMP-dependent protein kinases were purified as described (JBC 245, 12427, 1979) and antisera were raised in rabbits. Antibodies were purified by using cross-linked R<sup>I</sup> and R<sup>II</sup> sepharose (Fed. Proc. 41, 1304, 1982).
- 2) Immunocytochemistry of R<sup>I</sup> and R<sup>II</sup> in MCF-7 and MDAMB-231 cells were carried out as described (Hand Book of Exp. Pharm. 58, 333, 1982; Science, 211, 407, 1981).
- 3) The cross-reactivity and specificity of R<sup>I</sup> and R<sup>II</sup> antibodies were determined as described (JBC, 245, 12427, 1979; PNAS, 78, 653, 1981).
- 4) Other techniques used were standard methods.

Major Findings:

- 1) An indirect immunofluorescence technique was utilized to determine the intracellular distribution of R<sup>I</sup> and R<sup>II</sup> of cAMP-dependent protein kinases in hormone dependent estrogen receptor positive MCF-7 cells and hormone independent estrogen receptor negative MDAMB-231 cells.
- 2) Specific antibodies toward bovine skeletal muscle R<sup>I</sup> and bovine heart muscle R<sup>II</sup> were generated in rabbits. The cross-reactivity of bovine anti-R<sup>I</sup> and R<sup>II</sup> with human breast cancer cells were demonstrated by using sensitive radioimmunoassay and immunoprecipitation.
- 3) The antibodies of R<sup>I</sup> and R<sup>II</sup> were purified to homogeneity by using cross-linked-immunoabsorbent affinity chromatography. The cross-linking of R<sup>I</sup> and R<sup>II</sup> sepharose prevented the leakage of R<sup>I</sup> and R<sup>II</sup> subunits during the elution of anti R<sup>I</sup> and R<sup>II</sup> antibodies. From original anti-R<sup>I</sup> and anti-R<sup>II</sup> antisera approximately 500 and 200-fold purifications were obtained, respectively. The affinity purified antibodies were of IgG nature as identified by SDS gel electrophoresis and specific immunoabsorption analysis



analysis with goat anti-rabbit IgG and protein A-sepharose.

4) During initial stages of growth of MCF-7 cells both  $R^I$  and  $R^{II}$  were found in the nuclear area of dividing cells. In the nucleoli of cells, only  $R^{II}$  but not  $R^I$  was detected suggesting that  $R^{II}$  may be specifically involved in the chromatin directed events in MCF-7 cells.  $R^I$  was also found in the cytoplasmic area of MCF-7 cells. When cells formed characteristic rosette at three days of growth, the intensity of immunofluorescence of both  $R^I$  and  $R^{II}$  was dramatically increased in the nuclear area of MCF-7 cells. When cells formed monolayer, the immunofluorescence of  $R^{II}$  was redistributed to the cytoplasm and plasma membrane region of cells whereas the immunofluorescence of  $R^I$  was decreased dramatically in all areas of cells. These results suggest specific nuclear compartmentalization of  $R^{II}$  and differential nuclear directed roles of  $R^I$  and  $R^{II}$  of cAMP-dependent protein kinases in MCF-7 cells.

5) Experiments carried out by using unpurified antiserum (40% ammonium sulfate fraction of antiserum) of catalytic subunit (C) of cAMP-dependent protein kinase suggest that during different phases of growth of MCF-7 cells, the C was localized in the nuclei and cytoplasmic area of cells. After three days of growth of MCF-7 cells, C was localized on the nuclear membrane and with the microtubular filaments network of cells as the fluorescence pattern was microtubular filamentous type in the peripheral area of nuclei. Further experiments are required to prove the specificity of fluorescence and cross-reactivity by using affinity purified antibodies.

6) Besides the presence of  $R^I$  (47K protein) in MCF-7 cell soluble fraction, the analysis of cyclic AMP receptor proteins by  $8N_3$ -cAMP ( $^{32}P$ ) photo-affinity labelling revealed the presence of 50K isoprotein of type II cAMP-dependent protein kinase.

7) The subcellular distribution of  $R^I$  and  $R^{II}$  was also studied in hormone independent estrogen receptor negative MDAMB-231 human breast cancer cells. These cells are cylindrical in nature and potentially tumorigenic in nude mouse. During initial phase of growth of these cells,  $R^{II}$  was intensely localized in the nucleoli and mitotic chromosomes. The immunofluorescence of  $R^I$  was not observed either on nuclei or dividing chromosomes.

$R^I$  and  $R^{II}$  were also localized in the cytoplasmic area of MDAMB-231 cells. After 3 days of growth, the immunofluorescence  $R^I$  and  $R^{II}$  were not increased in the nuclear area of MDAMB-231 cells as was observed in the MCF-7 cells. However, after 5 days of growth, the immunofluorescence of  $R^{II}$  was increased in the nucleoli and peripheral area of nuclei. Immunofluorescence of  $R^I$  was increased slightly in the nuclei and cytoplasmic area of cell. After 7 days, the immunofluorescence of  $R^{II}$  and  $R^I$  were decreased slightly in all areas of cells. No

redistribution of staining of  $R^{II}$  was occurred into the plasma membrane and cytoplasmic region of MDAMB-231 cells as was observed in the MCF-7 cells. These results suggest that specific compartmentalization of  $R^{II}$  subunit occurs on the mitotic spindles of MDAMB-231 cells in estrogen hormone independent cells. The immunological studies suggest that estrogen receptor negative cells contain both type I and type II cAMP-dependent protein kinases.

Significance to Biomedical Research and the Program of the Institute:

The elucidation of the specific nucleoli and chromosomal compartmentalization of  $R^{II}$  and differential nuclear roles of  $R^I$  and  $R^{II}$  of cAMP-kinases in hormone-dependent and -independent human breast cancer cells may provide a new understanding into the initial events of growth of malignant cells. These studies raise the possibility whether similar distribution and compartmentalization of cAMP kinases exist in hormone responsive and hormone unresponsive human breast tumors.

Proposed Course of Research: 1) Since both hormone responsive MCF-7 and hormone independent MDAMB-231 cells have shown specific compartmentalization of  $R^{II}$  in nucleoli and chromosomes, studies are under active investigation to identify which form of  $R^{II}$  is present in nuclei during different phases of growth of cells and to investigate whether the presence of these kinases is specifically involved in the phosphorylation of endogenous proteins are currently underway. 2) Specific anti- $R^I$  and  $R^{II}$  antibodies will be utilized to determine the redistribution of cAMP-dependent protein kinases under various hormonal stimulus in MCF-7 and MDAMB-231 cells growing in vitro and in athymic nude mouse in vivo. 3) Specific affinity purified antibodies will be prepared against catalytic subunit of cAMP-kinase to determine its distribution, function, and relationship with the  $R^I$  and  $R^{II}$  of MCF-7 and MDAMB-231 cells.

Publications:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08278-01 LPP
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Migration of capillary endothelium by angiogenesis effectors		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: G. Alessandri K. Raju Other: P.M. Gullino	Visiting Fellow Staff Fellow Chief, LPP	LPP, NCI LPP, NCI LPP, NCI
COOPERATING UNITS (if any)		
LAB/BRANCH Laboratory of Pathophysiology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NIH, NCI, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0
CHECK APPROPRIATE BOX(ES)		
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
A new method has been developed that allows measurements of migration induced by angiogenesis effectors on capillary endothelium. <u>Angiogenesis</u> effectors were found to induce substances in the corneal tissue that mobilize <u>capillary endothelium</u> . Without the intermediary action of the cornea none of the angiogenesis effectors tested were able to mobilize endothelium except the Heparin + copper complex.		

Objective:

To elucidate the mechanism of angiogenesis.

Project Description:

Methods: A 0.1 mm gelatin layer was poured on the bottom of a petri dish. A 2.0 mm agarose was layered above the gelatin. Three holes 3 mm in diameter were punched into the agarose. Capillary endothelium was placed into the central hole, the test material was located in one lateral hole and the control material in the opposite hole. After 18 hr the endothelium migrates toward the chemotactic stimulus and forms an asymmetric hole. The asymmetry is measured and converted into a migration index.

Major Finding: The complex Heparin + copper appears to be the trigger that mobilizes endothelium during angiogenesis.

Significance to Biomedical Research and the Program of the Institute: New formation of vessels is indispensable for tumor growth. The understanding of angiogenesis opens the opportunity to interfere with neovascularization and, therefore, tumor growth.

Proposed Course of Research: To complete the project.

Publication:

Ziche, M. and Gullino, P.M. Angiogenesis and neoplastic progression in vitro. JNCI, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08279-01 LPP									
PERIOD COVERED October 1, 1981 to September 30, 1982											
TITLE OF PROJECT (80 characters or less)  Effect of proline analogs on normal and neoplastic breast epithelium											
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COOPERATING UNITS (if any) None											
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SECTION NCI, NIH, Bethesda, MD 20205											
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<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS	<input checked="" type="checkbox"/> x									
SUMMARY OF WORK (200 words or less - underline keywords)  <p>             Cis-hydroxyproline has been found to arrest the growth of rat mammary adeno-              carcinomas in vivo in the absence of general toxicity. The proline analog              was shown to be highly selective in blocking the production of <u>type IV</u>  <u>collagen</u> by the tumor cells in culture indicating that synthesis of this              protein is essential for the growth and/or survival of such tumors. These              results have been confirmed with two additional <u>proline analogs</u>, <u>azetidine</u>  <u>5-carboxylate</u> and <u>4-thioprolin</u> which are selective inhibitors of collagen              synthesis and which block the growth of mammary adenocarcinoma cells in              culture and in vivo. Azetidine 5-carboxylate and thioprolin caused tumor              regression after treatment of animals with 50 or 100 mg/kg SC, twice daily              for 10 days. Histological examination revealed no pathological effects on              cartilage, skin, intestines, liver, bone or spleen at these dosages. Nor              was the growth of the animals affected. The <u>tumor regression</u> may have been              mediated via effects of these <u>proline analogs</u> on collagen synthesis since              the relative amount of collagen in the treated vs control tumors was              reduced.           </p>											



## Project description:

Methods employed: Proline analogs were screened for their effects on collagen synthesis and cell growth using primary cultures of normal or tumor epithelium. Collagen production by the cultures was assessed on the basis of the relative amount of  $^3\text{H}$ -lysine incorporated into a collagenase sensitive form or on the basis of the amount of 4-hydroxyproline or hydroxylysine formed in the presence or absence of the analogs. Effects of the analogs on tumor growth was determined by measuring the change in tumor volume over the time course of treatment. Ultra-structural changes in the tumors was evaluated by electron microscopy.

Major findings: 1. Cis-hydroxyproline (CHP) blocks collagen secretion by myoepithelial cells and causes the regression of rat mammary adenocarcinomas. CHP has been shown to inhibit the growth of mammary adenocarcinoma and normal mammary epithelial cells in vivo and in vitro. Carcinomas which lack the myoepithelial cell type were not affected by CHP. Electron microscopic analysis of adenocarcinomas from control and CHP treated animals revealed that the proline analog causes a defective basal lamina to form in the myoepithelial cells. CHP treatment apparently resulted in the production of defective collagen that could not be processed properly. This material accumulates in the golgi and no evidence of actively extruded lamina was seen. With tumors from untreated animals, material with the same electron density as basal lamina was actively being deposited. These results confirm our conclusions reached from immunological and biochemical analyses that the myoepithelial cells are responsible for basal lamina deposition. No evidence of basal lamina production by the epithelial cells was found. 2. Other proline analogs have been shown to selectively block collagen production and cause the growth arrest or regression of mammary adenocarcinomas. Azetidine 5-carboxylate (Az), the 4 membered ring analog of proline, has been found to be one of the most selective inhibitors of collagen production in mammary cells, being 7 times more selective in differentially blocking collagen synthesis in pulse labeling experiments than CHP. This compound also blocks tumor cell growth in vivo and in vitro more efficiently than CHP. The dose yielding 50% inhibition of growth in culture is 1/2 that required for CHP. In vivo, AZ blocks tumor growth at a concentration 1/2 that that needed for CHP induced inhibition. The AZ effect on tumor growth may be via inhibition of collagen synthesis since the relative amount of collagen in tumors from treated animals is 1/4 that of controls. Thioproline (TP), the proline analog in which sulfur replaces carbon in the #4 position of the ring, is also an effective inhibitor of collagen production and tumor cell growth in culture. TP also blocks the growth of the tumor cells in vivo. Two additional proline analogs have been evaluated only in cultured mammary tumor epithelium to date. One of these, 3,4-dehydroproline, was a poor inhibitor of collagen synthesis or cell growth. Total protein synthesis and collagen synthesis were inhibited to about the same extent by this analog. Pipecolic acid, the 6 membered ring analog of proline was about equivalent to AZ in its specificity of inhibition of collagen production in cultured cells. Unlike AZ this compound did not inhibit cell growth in the cultures. We tentatively conclude that pipecolic acid incorporation into collagen produces a protein that is rapidly degraded and does not accumulate in the golgi apparatus and therefore does not kill the cells.

Significance to biomedical research and the program of the institute: The formation of the basal lamina with its constituent collagen appears to be a fundamental and essential part of the proliferative process of normal epithelium of the mammary gland and by differentiated tumors derived from it. Because in the adult the production of collagen is not extensive, tumors making collagen can be differentially killed by blocking collagen formation (or secretion) with proline analogs in the absence of toxic effects on normal tissues.

Proposed course of future research: Additional studies will be performed to further delineate the requirement for collagen synthesis in mammary tumors and to determine how this requirement is obviated as adenocarcinomas progress to carcinomas.

**Publications:**

Strum, J.M., Lewko, W.L. and Kidwell, W.R.: Structural alterations within Nitrosomethylurea-induced mammary tumors after in vivo treatment with cis-hydroxyproline. Lab. Invest. 45: 347-354, 1981.

## LABORATORY OF MATHEMATICAL BIOLOGY

### SUMMARY

October 1, 1981 through September 30, 1982

#### Summary Report

The activities of the Laboratory of Mathematical Biology (LTB) fall into several broad areas: membrane biophysics, immunology, macromolecular configurations, kinetics of metabolic systems and computational and modeling methodology. Most of the work is theoretical, but experiments are also carried out in the laboratory. Much of the theoretical work and modeling is done in collaboration with experimental groups at NIH and elsewhere.

The studies in the Section on Membrane Structure and Function follow two directions: 1) the insertion and organization of molecules (proteins, lipids) in membranes, and 2) the cell biology and pharmacology of lipid vesicles. We use spectroscopic techniques (fluorescence, circular dichroism) to study lipid-protein interactions, and measure conductance across planar black lipid membranes (BLMs) to approach questions of membrane protein topology.

The following systems are investigated: 1) Interactions of tubulin with lipid vesicles at the lipid phase transition (Weinstein, Klausner, and Blumenthal). 2) The effect of charge clusters and the membrane potential on the disposition of membrane proteins. We are carrying out BLM studies with the hepatic asialoglycoprotein receptor and with melittin (Blumenthal, Klausner, Weinstein, Kempf, and vanRenswoude). 3) Translation, translocation and folding of ovalbumin using endoplasmic reticulum membranes (Klausner, vanRenswoude, Kempf, Blumenthal, and Weinstein). 4) Fusion of lipid bilayers induced by such proteins as clathrin and tubulin using resonance energy transfer between two lipid fluorophores incorporated into the same bilayer (Blumenthal, Morris, Kumar, Steer, Henkart, Weinstein, and Klausner). 5) Lateral organization of membrane components on cell surfaces in epithelial cells (Dragsten, Blumenthal, and Hendler).

Work on liposomes has centered on three ways to direct liposome-encapsulated agents to tumor targets: (1) Antibody-mediated targeting to tumor cells, with immunoglobulin attached to the liposome covalently or through binding sites; (2) Temperature-sensitive liposomes, so designed as to "self-destruct" and release encapsulated drug selectively in a tumor; (3) Compartmental delivery of liposomes and their contents to lymph node micro-metastases (Weinstein).

Work has begun on the in vivo use of monoclonal antibodies for diagnosis and treatment of tumors. The initial aim has been to delineate pharmacokinetic principles in well-defined, reproducible systems. The results are being used to design optimal agents for gamma camera imaging and, potentially, for therapy (Weinstein).

The development and application of mathematical techniques for describing receptor clustering on the plasma membrane continued (C. DeLisi). The results were incorporated into a theory of the response of sensitized basophils and

mast cells which were proposed to explain, in terms of the physical chemistry of cell surface events, the biochemical pathway (histamine release, specific desensitization, non-specific desensitization) selected by the cell. Aside from predicting the parameters of importance in the control of this choice, the theory also provides an explanation for the wide range of qualitative differences observed in dose response curves. The work is in collaboration with R. Siraganian, NIDR.

Experimental applications of the theory of cell surface events were extended to IgG complexes interacting with Fc receptors on macrophages (DeLisi, Dower, Segal). This work is relevant to immune complex effector mechanisms, and may also provide insight into the analysis of data on a number of other systems, such as those involving binding of hormones or neurotransmitters to cell surface receptors. J. Hiernaux and P. Baker (NIAID) continued their experimental work on immune response dynamics that was initiated as a consequence of our theoretical models of certain aspects of systemic regulation of the immune response. The model is intended primarily for T-independent antigens, but a number of regulatory features are expected to be general. Several predictions including oscillations in antibody affinity, are now being tested experimentally.

The work on assays has been extended to liquid column chromatography (DeLisi, Hethcote). We have developed a non equilibrium theory which includes diffusion, and have obtained analytic expressions relating elution profile characteristics to thermodynamic and kinetic parameters. The equations are currently being applied and tested by J. Inman (NIAID) and I. Chaiken (NIAMDD).

Research within the laboratory also encompasses biological macromolecules and their properties. Stabilities of macromolecular conformations are determined by interatomic interactions; the relative importance, for proteins, of various classes of interactions, short range and long range, is being assessed in detail (Jernigan and Miyazawa). Short and medium range interactions appear to determine locations of regular  $\alpha$ -helices. Several simple models of long range intramolecular interactions have been formulated to facilitate investigations of protein folding pathways. Monte Carlo generations of protein conformations led to development of a simpler method, similar to a helix-coil theory, to describe the folding process. Methods for "trapping" folding intermediates are necessary because they are usually not present in appreciable concentrations. From calculated probabilities of each residue in the native conformation at each stage of folding, folding-unfolding pathways can be constructed. Typically, folding is observed to proceed by growth from only a few centers within the molecule.

More detailed calculations of conformational energies, requiring evaluation of all interatomic distances, are feasible for protein fragments or small proteins. Such calculations have lead to postulation of new binding sites for lysozyme (Pincus, Smith-Gill and Scheraga). Experimentally, it was found that the substrate displaced the site specific monoclonal antibody, thereby verifying the calculated binding sites. Similar calculations are being applied to the variable regions of the antigen combining site of myeloma immunoglobulins; these indicate substantial conformational variability for changed residues (Pincus, Potter and Feldmann). Membrane embedded proteins are



being studied; in particular their preferred conformations within the membrane are sought. Mellitin and the leader peptide sequences, which are those portions of proteins assisting in secretion through membranes, are being considered (Pincus, Klausner, Kempf, vanRenswoude and Blumenthal). They are a sequentially diverse class of peptides; however, their similar functions would indicate that conformational similarities are likely. It has been found that they are probably substantially in the  $\alpha$ -helix conformation within membranes, in agreement with several experimental findings. In particular, the calculated conformation of mellitin was in excellent overall agreement with the reported X-ray crystal form. This theoretical approach is now being adapted to proteins in water, particularly ribonuclease A (Pincus, Gerewitz and Carty), to obtain folding nucleation sites. Recent experiments indicate the inhibition of folding with a protein fragment, comprising the calculated nucleation site, to confirm these results.

Modeling of the endocrine system has continued with emphasis on lipoproteins, the glucose-insulin system and receptors (Berman, Wastney, Covell, and Chu). Further extensions of the lipoprotein system have been made by studies of triglyceride kinetics and  $\alpha_2$ - and  $\beta$ -VLDL apoB kinetics (Berman and Chu), the latter in collaboration with the Seattle group (Drs. Foster and Hazzard). Combined studies, involving both TG and apoB in the same patient are being considered.

Detailed modeling of ketone bodies has also been carried out (Wastney and Berman) in collaboration with Dr. S. Hall of Ottawa, who carried out the experiments. This relates to other modeling carried out by this group on intermediate metabolism. Studies on insulin secretion in various populations and its variation as a function of glucose load have also been studied (Berman and Covell).

The modeling techniques have been further extended by adding a number of new features in Conversational SAAM (CONSAM) on our VAX 11/780 computer (Berman, Boston, Kravitz, and Joffe). This is a continuing project as the power of the SAAM program is being extended.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  ZO1 CB 08300-10 LTB																																	
PERIOD COVERED  October 1, 1981 to September 30, 1982																																			
TITLE OF PROJECT (80 characters or less)  SAAM, Modeling and Applications																																			
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																																			
PI: <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">Mones Berman, Ph.D.</td> <td style="width: 33%;">Chief</td> <td style="width: 33%;">LMB NCI</td> </tr> <tr> <td>Ira Schwartz, Ph.D.</td> <td>Staff Fellow</td> <td>LMB NCI</td> </tr> <tr> <td>Martha Chu, Ph.D.</td> <td>Visiting Fellow</td> <td>LMB NCI</td> </tr> <tr> <td>David Covell, Ph.D.</td> <td>Staff Fellow</td> <td>LMB NCI</td> </tr> <tr> <td>Meryl Wastney, Ph.D.</td> <td>Visiting Fellow</td> <td>LMB NCI</td> </tr> <tr> <td>Raymond Boston, Ph.D., Dept. of Agriculture,</td> <td colspan="2">LaTrobe Univ., Australia</td> </tr> <tr> <td>Peter Greif, M.D.</td> <td colspan="2"></td> </tr> <tr> <td>Saul Kravitz</td> <td colspan="2"></td> </tr> <tr> <td>Sandor Joffee</td> <td colspan="2">LMB NCI</td> </tr> <tr> <td>Naomi Sager, Ph.D.</td> <td>Linguistic String Lab.,</td> <td>Courant Inst, N.Y. Univ.</td> </tr> <tr> <td>David Walker,</td> <td>Census Bureau,</td> <td>Washington, D.C.</td> </tr> </table>			Mones Berman, Ph.D.	Chief	LMB NCI	Ira Schwartz, Ph.D.	Staff Fellow	LMB NCI	Martha Chu, Ph.D.	Visiting Fellow	LMB NCI	David Covell, Ph.D.	Staff Fellow	LMB NCI	Meryl Wastney, Ph.D.	Visiting Fellow	LMB NCI	Raymond Boston, Ph.D., Dept. of Agriculture,	LaTrobe Univ., Australia		Peter Greif, M.D.			Saul Kravitz			Sandor Joffee	LMB NCI		Naomi Sager, Ph.D.	Linguistic String Lab.,	Courant Inst, N.Y. Univ.	David Walker,	Census Bureau,	Washington, D.C.
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SUMMARY OF WORK (200 words or less - underline keywords)  Continuing development of a <u>computer system (SAAM)</u> for the simulation, analysis and modeling of bio-kinetic systems. Further development of a <u>conversational mode of operation</u> , increased versatility of applications and <u>automation in modeling</u> are in progress. Analysis of various <u>kinetic</u> and metabolic systems by the use of <u>mathematical models</u> carried out with other investigators.																																			

## Project Description

**Objectives:** To develop a general purpose computer program for modeling bio-kinetic systems that may readily be used by investigators not sophisticated in mathematics or programming. Initiated in 1959, the program continues to be expanded and revised as new features are added.

Analysis of data on metabolic systems, and further development of modeling theory and techniques through the applications of the SAAM computer program.

**Major Findings:** Through the visits of Dr. Ray Boston from LaTrobe University, Australia, new features have been incorporated in conversational SAAM (CONSAM). These include the abilities to interface with the least squares convergence procedures and to modify them to compensate for non linearities. The differential equations solution algorithms are also being expanded to take advantage of the latest state of the art in this area (Schwartz).

The SAAM programs are being applied to a variety of problems. A model was constructed for the kinetics of warfarin in dogs (Covell); HDL apo A kinetics were studied in the dog in collaboration with Dr. A. Scanu (Univ. of Chicago) (Chu), and in monkeys -- in collaboration with Dr. Peter Herbert (Unif. of Rhode Island) (Chu). Various other transport problems have also been modeled in collaboration with other investigators, or as part of a training activity.

A pilot study was initiated this year to develop data bases for various metabolic systems to serve as bases for the development and testing of models. The data bases will be constructed partly from available data and partly from literature data. Formatting of the literature data is now in progress in collaboration with Dr. Naomi Sager, who heads a computer linguistics group at New York University, NY.

**Significance to Biomedical Research and the Program of the Institute:** The methodology of modeling is most essential for studying the behaviour of systems for both normal and abnormal states. The SAAM computer program has been a most essential tool in the modeling of various systems described elsewhere in this laboratory report. Its wide use in other centers in the United States and elsewhere is further evidence of its value in biomedical research.

**Proposed Course:** This is a continuing process for the development of theory and for the application of modeling techniques.

## Publications:

Boston, R., Greif, P., and Berman, M.: CONSAM-Conversational SAAM as a Modeling Tool. In Berman, M., Grundy, and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Boston, R.C., Greif, P.C., and Berman, M.: Conversational SAAM - An interactive program for kinetic analysis of biological systems. Computer Programs in Biomedicine. 111-119, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08303-10 LTB
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Movement of Molecules in Membranes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: R.P. Blumenthal, Ph.D. Chief, Membrane Structure and Function Section LTB NCI

OTHERS: John N. Weinstein, M.D., Ph.D. Investigator LTB NCI

COOPERATING UNITS (if any)

P. Henkart, Ph.D.	IB NCI
J. Handler, M.D.	LKEM NHLB
M.C. Fishman, M.D.	LDN, NICHD
R.D. Klausner, M.D.	LBM, NIAMDD

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Membrane Structure and Function Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.5	OTHER: 0
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CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

We study the organization and changes in organization of membrane components (lipids and proteins), both in the lateral and in the perpendicular direction. (1) We follow the insertion of a protein into a preformed lipid bilayer (either in the form of a planar bilayer or of a lipid vesicle), and study the factors which determine the protein's orientation. We measure electrical properties of Black Lipid Membranes (BLM) to study: (a) mechanisms of ion transport; (b) properties of transport systems isolated from natural cell membranes; (c) mechanisms of cytotoxicity; (d) the effect of the membrane potential on the disposition of membrane proteins. (2) We have developed model systems in which fusion of phosphatidylcholine bilayers is induced by such proteins as tubulin and clathrin. We study this fusion process using an assay involving resonance energy transfer between two fluorophores incorporated into the vesicle bilayer. (3) We observe lateral organization and movement of fluorescently - labelled molecules on cell surfaces by fluorescence microscopy, and assess diffusion coefficients by the technique of fluorescence recovery after photobleaching (FRAP). We study the mechanism by which asymmetry is maintained in epithelia.

Project Description:

Objectives: To study the physical mechanisms of ion transport in reconstituted membranes. To develop the lipid bilayer membrane as an assay for transport systems isolated from natural cell membranes. To study mechanisms of cytotoxicity. To study the effect of the membrane potential on the disposition of membrane proteins. To study the role played in cell membranes by the mobility and distribution of cell surface receptors. To study the physiological significance of domains of lipid in membranes. To study factors which constrain the movement of membrane protein and lipids to specific areas of the cell surface. To study the mechanism of membrane fusion.

Methods Employed: The bilayer membranes are formed from natural membrane extracts, oxidized cholesterol, or pure lipids in a aperture between two electrolyte solutions. The electrical properties of the membranes are measured before and after application of an activating factor. Lipids vesicles are formed by bath sonication, probe sonicate and reverse - phase evaporation. Leakage from vesicles is assayed by measuring the increase in fluorescence as vesicle-encapsulated self-quenched carboxyfluorescein is released into the medium and diluted. Spectroscopic changes upon interaction of proteins with lipid vesicles are studied by fluorometry and circular dichroism. Diffusion of fluorescently labelled molecules (proteins, lipids, carbohydrates) on cell surfaces is measured by the technique of fluorescence photobleaching recovery. Other techniques to measure movement of molecules in membranes are fluorescence polarization, fluorescence energy transfer, fluorescence life time heterogeneity analysis, fluorescence stopped-flow kinetics and  $^{31}\text{P}$  NMR.

Major Findings:Protein - Bilayer interactions:

1) Tetanolysin, a hemolytic toxin, exerts its lytic effect by perturbing the lipid bilayer structure as shown by conductance step size analysis and by temperature and concentration dependence of the conductance increase in BLMs. 2) Stable tubulin - vesicle complexes are formed by incubating the protein with dipalmitoyl phosphatidylcholine at the lipid phase transition temperature. 3) The interactions is accompanied by conformational changes in the protein and involves some sequestration of the protein in the bilayer. 4) The tubulin - vesicles complexes are intact; they contain a closed internal space. The structure of the lipid bilayer does not appear to be affected by the interaction. 5) Vesicle-tubulin does bind colchicine and microtubule - associating protein, but does not copolymerize with tubulin into microtubules. 6) At pH 6.5 and below clathrin coat protein induces a voltage - dependent increase ion conductance across a black lipid membrane and induces leakage of contents from phosphatidylcholine vesicles. Stable clathrin - phospholipid complexes are formed as shown by density gradient centrifugation. Coated vesicles do not interact with lipid bilayers.



#### Fusion:

1) Addition of millimolar concentrations of  $\text{Ca}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Co}^{2+}$ , but not  $\text{Mg}^{2+}$  caused the vesicle-tubulin complexes to fuse as shown by electron microscopy, increased trapped volume, and changes in resonance energy transfer between two fluorescent lipid probes incorporated into the same vesicle. There was no loss of internal aqueous contents from the vesicle-tubulin complexes during  $\text{Ca}^{2+}$  induced fusion. 2) At pH 6.5 and below clathrin induced dioeyol phosphatidylcholine bilayers to fuse.

Significance for Biomedical Research and the Program of the Institute: Mobility, distribution and expression of cell surface components are considered to have important implications for cell transformation and for many aspects of the physiology of normal and tumor cells.

Proposed Course: A) The BLM will continue to be used as an assay for conductance - inducing materials from cells. The notion of voltage - dependent assembly of proteins in membranes will be further explored with different lipid composition, and with peptides with specific charge cluster distribution. We hope to study the voltage - dependent effects of leader sequences on lipid bilayers, and to develop an in vitro protein assembly system using the BLM. B) Further studies on the mechanism of membrane fusion will involve manipulation of the lipid composition, stopped-flow kinetics,  $^{31}\text{P}$ -NMR and freeze - cleavage. We hope to fuse vesicle - tubulin complexes with BLMs, examine whether  $\text{Ca}^{2+}$  binding proteins could lower the  $\text{Ca}^{2+}$  requirement for fusion, and fuse the complexes with secretory granules. We plan to identify an intermediate fusion complex (inverted micelle) using our fluorescence energy transfer techniques. We plan to identify factors that stabilize such an intermediate in tight junctions.

#### Publications:

Klausner, R.D., Kumar, N., Weinstein, J.N., Blumenthal, R., and Flavin, M.: Interaction of tubulin with phospholipid vesicles I: Association with vesicles at the phase transition. J. Biol. Chem. 256: 5879-5885, 1981.

Kumar, N., Klausner, R.D., Weinstein, J.N., Blumenthal, R., and Flavin, M.: Interaction of tubulin with phospholipid vesicles II: Physical changes of the protein. J. Biol. Chem. 256: 5886-5889, 1981.

Weinstein, J.N., Klausner, R.D., Innerarity, T., Ralston, E., and Blumenthal, R.: Phase transition release: A new approach to the interaction of proteins with lipid vesicles: Application to lipoproteins. Biochim. Biophys. Acta. 647: 270-284, 1981.

Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.N and Blumenthal, R.: Carboxyfluorescein as a probe for liposome-cell interactions: Effect of impurities and purification of the dye. Biochim. Biophys. Acta. 649: 133-137, 1981.

Dragsten, P.R., Blumenthal, R., and Handler, J.S.: Membrane asymmetry in epithelia: Is the tight junction a barrier to diffusion in the plasma membrane? Nature. 294: 718-722, 1981.



Blumenthal, R., and Klausner, R.D.: The interaction of membrane proteins with black lipid membranes. In Poste G., and Nicholson G.L. (Eds.): Cell Surface Reviews. 1982, Vol. 8. in press

Blumenthal, R.P., Klausner, R.D., and Weinstein, J.N.: Voltage-dependent changes of a membrane protein in lipid model membranes: Studies with the hepatic asialoglycoprotein receptor. Biophys. J. 37: 122-124, 1982.

Blumenthal, R.: Transport mechanisms for radiotracers. In Colombetti, L.G. (Ed.): Advances in radiopharmacology. 1: 150-156, 1981.

Kempf, C., Klausner, R.D., Weinstein, J.N., van Renswoude, J., Pincus, M., and Blumenthal, R.: Voltage-dependent transbilayer orientation of melittin. J. Biol. Chem. 257: 2469-2476, 1982.

Dragsten, P.R., Handler, J.S., and Blumenthal, R.: Fluorescent Membrane Probes and the Mechanism of Cellular Asymmetry in epithelia. Federation Proc. 41: 48-52, 1982.

Weinstein, J.N., Leserman, L.D., Henkart, P.A., and Blumenthal, R.: Antibody-mediated targeting of liposomes. In Gregoriadis, G., and Papahadjapoulos, D. (Eds): Targeting of Drugs. New York, Plenum, 1982 in press.

Weinstein, J.N., Blumenthal, R., Kempf, C., van Renswoude, J., and Klausner, R.D.: Charge clusters and the orientation of membrane proteins. J. Membr. Biol. 66: 203-212, 1982.

Blumenthal, R.: Membrane transport: In Colombetti, L. (Ed.): Radiotracers in Biology and Medicine. Vol. IX 1982 in press.

van Renswoude, J., Kempf, C., Weinstein, J.N., Blumenthal, R., and Klausner, R.D.: Folding of ovalbumin: Exposure and sequestration of the N-terminus as the functional leader sequence during biosynthesis. J. Biol. Chem. 1982, in press.

Steer, C.J., Klausner, R.D., and Blumenthal, R.: Interaction of liver clathrin coat protein with lipid model membranes. J. Biol. Chem. 1982, in press.

Klausner, R.D., Berman, M., Blumenthal, R., Weinstein, J., and Caplan, S.R.: Compartmental analysis of light-induced proton movement in reconstituted bacteriorhodopsin vesicles. Biochemistry. 1982 in press.

Dragsten, P.R., Handler, J.S., and Blumenthal, R.: Asymmetry in epithelial cells: Is the tight junction a barrier to lateral diffusion in the plasma membrane? In Bolis, S., Hoffman, J.F., and Giebisch, G. (Eds.): Membranes in Growth and Development. New York, Liss, in press.

Blumenthal, R., Ralston, E., Dragsten, P., Leserman, L.D., and Weinstein, J.N.: Lipid vesicle-cell interactions: Analysis of a model for transfer of contents from adsorbed vesicles to cells. Membrane Biochemistry, 1982, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08306-10 LTB
PERIOD COVERED  October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Kinetic Modeling of Human Plasma Lipoprotein Metabolism		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Mones Berman, Ph.D. Chief LMB NCI		
OTHERS: Meryl Wastney, Ph.D. Visiting Fellow LMB NCI Martha Chu, Ph.D. Visiting Fellow LMB NCI Scott Grundy, M.D. Veterans Administration, San Diego, CA Waldo Fisher, M.D. Univ. of Florida, Gainesville, FL Charles Schwartz, M.D. Veterans Administration, Richmond, VA Claude Malmendier, M.D. Univ. of Brussels, Belgium		
COOPERATING UNITS (if any) VA Hospital, Richmond VA; VA Hospital, San Diego, CA; UCSD School of Medicine, Univ. of Fla.; Univ. Brussels		
LAB/BRANCH  Laboratory of Mathematical Biology		
SECTION  Office of the Chief		
INSTITUTE AND LOCATION  NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  1.3	PROFESSIONAL:  1.3	OTHER:  0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <p> <u>Kinetic models</u> of plasma <u>apoproteins</u>, <u>cholesterol</u> and <u>triglyceride</u> are          being constructed based on data from experiments in <u>man</u>. The models are used          to integrate plasma <u>lipoprotein</u> interactions with enzymes and receptors and          to provide a better understanding of <u>plasma lipoprotein synthesis and metabo-</u>  <u>lism in health and disease</u>. The models are particularly useful for the          rigorous testing of hypotheses, the design of experiments, and the quantifi-          cation of the effects of various perturbations.       </p>		

Project Description:

Objectives: To develop a qualitative and quantitative understanding of lipoproteins metabolism in man and to identify abnormalities and drug effects through the modeling of apoprotein, cholesterol, and triglyceride kinetics. Because of the need for diverse extensive data on a variety of patients, collaboration with several experimental groups is maintained.

Methods Employed: Mathematical modeling is the tool used for the integration and analysis of the data. This is performed with the help of the SAAM computer modeling program.

Major Findings: The modeling continues along various routes of the lipoprotein system. The kinetics of asialated and sialated LDL were examined (Wastney) and the kinetics of apoE in normal and type III patients were modeled (Chu). Although no definitive conclusions could be drawn from these kinetics, several interesting conjectures were advanced. In the case of apo E kinetics - it seems possible that the tracer data permit the identification of an E-III component in normal apoE and a change in affinity for apoE in type III patients.

The overall model for triglycerides (TG) kinetics (Zech, et al.) was further extended to include TG exchanges between HDL and VLDL and the incorporation of TG exchanges between HDL and VLDL and the incorporation of TG in newly synthesized IDL and LDL (Berman). The kinetics of cholesterol ester (Schwartz) were studied and the rates of exchange between the various plasma components determined. It appears that free cholesterol of HDL is the primary substrate for newly formed cholesterol ester.

A book on lipoprotein kinetics and modeling was edited and is now in the process of publication (Academic Press).

Significance to Biomedical Research and the Program of the Institute: Modeling is important to identify the abnormalities in metabolism responsible in hyperlipemias, diabetics, and other metabolic disorders, given a very complicated system with multiple interactions. It is hoped that by identifying the mechanisms responsible for abnormalities in lipid metabolism and their relations with intermediate metabolism, appropriate treatment can be more specifically geared to abnormalities. Clinically this is relevant to atherosclerosis and disorders associated with atherosclerosis such as cardiovascular disease, diabetes, cachexia, and other metabolic disorders involving lipids.

Publications:

Berman, M.: ApoC Kinetics. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Schwartz, C.C., Berman, M., Halloran, L.G., Swell, L. and Vlhacevic, Z.R.: Determination of Total Body Disposal in Man: Special Role of HDL Free Cholesterol. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Wastney, M.E., Riemke, R., Malmendier, C.L., and Berman, M.: Heterogeneity of Low Density Lipoprotein: Kinetic Analyses of Asialated Lipoproteins. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Berman, M., Beltz, W.F., Riemke, R., and Grundy, S.M.: VLDL-RG Exchange with HDL-TG In Vivo. In Berman, M., Grundy, S.M., and B.V. Howard (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Grundy, S.M., Mok, H.Y.I., Zech, L., and Berman, M.: Influence of nicotinic acid on metabolism of cholesterol and triglycerides in man. J. Lipid Research, 21: 760-774, 1980.

Le, N-A., Grundy, S.M., and Berman, M.: A Reduced Model for Very Low Density Lipoprotein Triglyceride Metabolism. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Berman, M., and Chu, M.: A Possible Interpretation of ApoE Kinetics in Man. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

Berman, M.: Kinetic Analysis and Modeling: Theory and Applications to Lipoproteins. In Berman, M., Grundy, S.M., and Howard, B.V. (Eds.): Lipoprotein Kinetics and Modeling. New York, Academic Press. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08320-07 LTB								
PERIOD COVERED <div style="text-align: center;">October 1, 1981 to September 30, 1982</div>										
TITLE OF PROJECT (80 characters or less)  <div style="text-align: center;">Macromolecular Conformations</div>										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 40%;">Robert L. Jernigan, Ph.D.</td> <td style="width: 30%;">Theoretical Physical Chemist</td> <td style="width: 15%;">LTB NCI</td> </tr> <tr> <td>OTHERS:</td> <td>Sanzo Miyazawa, Ph.D.</td> <td>Visiting Fellow</td> <td>LTB NCI</td> </tr> </table>			PI:	Robert L. Jernigan, Ph.D.	Theoretical Physical Chemist	LTB NCI	OTHERS:	Sanzo Miyazawa, Ph.D.	Visiting Fellow	LTB NCI
PI:	Robert L. Jernigan, Ph.D.	Theoretical Physical Chemist	LTB NCI							
OTHERS:	Sanzo Miyazawa, Ph.D.	Visiting Fellow	LTB NCI							
COOPERATING UNITS (if any)  <div style="text-align: center;">None</div>										
LAB/BRANCH  <div style="text-align: center;">Laboratory of Mathematical Biology</div>										
SECTION  <div style="text-align: center;">Office of the Chief</div>										
INSTITUTE AND LOCATION  <div style="text-align: center;">NCI, NIH, Bethesda, Maryland, 20205</div>										
TOTAL MANYEARS: <div style="text-align: center;">1.8</div>	PROFESSIONAL: <div style="text-align: center;">1.8</div>	OTHER:								
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;"><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td style="width: 33%;"><input type="checkbox"/> (b) HUMAN TISSUES</td> <td style="width: 33%;"><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS				
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER								
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS										
SUMMARY OF WORK (200 words or less - underline keywords)  <p> <u>Protein conformations</u> have been investigated by calculations which include either short and medium range or long range interactions. These include 1) predictions of <u>regular secondary regions</u>, with a method which does not depend on reported X-ray crystal forms, and 2) investigations of <u>folding pathways</u> of proteins with a simple model in which the native conformation is favored. In the first case, it is found that, similar to other secondary conformation prediction methods, only about 60% of the residues are favored in their native conformation, if only short and medium range interactions are included. The implication is that <u>long range interactions</u> modify intrinsically preferred conformations of significant numbers of residues. From the second calculations which include long range interactions, definite folding pathways are obtained for <u>pancreatic trypsin inhibitor</u>, <u>myoglobin</u>, <u>ribonuclease</u>, <u>concanavalin A</u>, and <u>lysozyme</u>. The <u>activated state</u> for the folding-unfolding process is found to correspond to the appearance of long range interactions. In all cases only one or two regions of growing nuclei are observed.         </p>										



Objectives: To develop theoretical methods adequate to determine the most significant macromolecular conformations. The principal molecules of interest are proteins. At the most elementary level, we wish to predict protein conformations from their sequences and to determine the relative importances of various intramolecular interactions. At present, methods are limited and are not expected to yield complete three dimensional conformations of large proteins. We would like to develop simple models to generate conformations important at different stages of folding and then construct folding pathways.

Methods Employed: Approximate methods for calculating conformational energies, based on electrostatic energies and hard sphere atomic representations, have been developed. These are utilized as input for methods to select best sets of regular secondary conformational regions.

Molecular conformations are generated for all extents of folding; this permits a detailed treatment of folding pathways, by considering most probable protein conformations in samples which are denatured to different extents. The folding-unfolding process is investigated with an idealized model employing approximate free energies. Intra-residue energies consist of an empirical energy taken from the observed frequency distributions in conformational angles ( $\phi, \psi$ ). Inter-residue interactions are simplified by assuming that there is an attractive energy operative only between residue pairs which are in close contact in the native structure. These are utilized in a "helix-coil like" model in which long range interactions are included only within native globules. Most probable native residues are indicated at all extents of folding. From these results, probable pathways through the folding-unfolding transition can be constructed.

Major Findings: The present secondary structure calculations yield results correct for about 60% of the residues in a protein. This is, however, not sufficient for making detailed statements about overall three dimensional protein conformations. In the simplified secondary energy calculations, a single set of energy parameters has been obtained to represent the mean field corresponding to a general globular state. Numerous points of flexibility, representing sites relatively indifferent to conformation, may provide substantial explanation of the errors manifested in such secondary structure predictions.

For this idealized model all protein folding transitions appear to be an "all-or-none" type; such behavior can be attributed to the highly specific long range interactions. It is found that turns and  $\alpha$  helices appear at an early stage but long range interactions, including those found in  $\beta$  sheets, usually appear near the maximum in the free energy as an activated state in a cooperative step. Results are similar to those obtained in more detailed Monte Carlo generations of conformations with volume exclusion. Most probable conformations are found to be relatively insensitive to the exact value of the contact energy parameter. Calculations were performed for trypsin inhibitor, myoglobin, lysozyme, concanavalin A and ribonuclease A. In all cases, one or two nuclei for growth of native regions were found.

Proposed Course: Effects of side chain-side chain interactions on calculated secondary region energies will be investigated. Previous methods have neglected these interactions. Secondary conformation calculations may serve as an initial guide in choosing initial conformations for considerations of possible long range interactions.

More detailed evaluations of the effects on the pathways of excluded volume and the strengths of intramolecular interaction energies will be pursued. More realistic energies will be developed in an attempt to include the possibilities of favorable non-native conformations. This should permit consideration of reported folded intermediates with shuffled disulfide bonds. Development of methods to treat tightly packed protein conformations is anticipated. Applications of theories of phase transitions of rod-like molecules are anticipated in treating polypeptide transitions to ordered, high density forms. In order to comprehend the appearance of favorable long range interactions, probabilistic studies of the effects of classes of interactions on ring closure are anticipated.

Publications:

Miyazawa, S., and Jernigan, R.L.: Equilibrium folding and unfolding pathways for a model protein. Biopolymers, in press.

Miyazawa, S., and Jernigan, R.L.: Most probable intermediates in protein folding-unfolding with a non-interacting local structure model. Biochemistry, in press.

Jernigan, R.L., and Miyazawa, S.: Equilibrium folding-unfolding pathways of model proteins: effect of myoglobin heme contacts. Biopolymers, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08323-07 LTB
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Assay Quantitation</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Charles DeLisi, Ph.D. <span style="float: right;">Chief, Theoretical Immunology Section</span> <span style="float: right;">LTB NCI</span>		
OTHERS: Herbert Hethcote, Ph.D. <span style="float: right;">Mathematics, Univ. of Iowa</span>		
COOPERATING UNITS (if any) John Inman Ph.D., Laboratory of Immunology, NIAID; Irwin Chaiken, Ph.D., Laboratory of Chemical Biology, NIAMD; Prof. Pierre Masson, Inst. Cellular Molec. Pathol., Brussels; Prof. Gregory Siskind, Div. of Immunol., Cornell Univ. Med. School.		
LAB/BRANCH <p style="text-align: center;">Laboratory of Mathematical Biology</p>		
SECTION <p style="text-align: center;">Theoretical Immunology Section</p>		
INSTITUTE AND LOCATION <p style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS: <p style="text-align: center;">0.7</p>	PROFESSIONAL: <p style="text-align: center;">0.7</p>	OTHER:
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) A theory has been developed to describe a <u>heterogeneous, non equilibrium</u> system in which <u>diffusion, transport</u> and <u>chemical reaction</u> are all occurring simultaneously. The variables entering the equations are: bead bound antigens, mobile antibodies that react specifically with the antigens but which have a distribution of rate and equilibrium constants for them, and mobile antigens which inhibit the antibody-bead bound antigen interaction. Characteristics of the <u>affinity</u> and <u>rate constant</u> distributions are related to characteristics of the elution profile by relatively simple expressions even for systems in which neither chemical equilibrium nor a local steady state has been established. The effect of movement in and out of the bead under <u>non ideal conditions</u> (activity coefficient different from unity) is included. An important aspect of the development is the theoretical relation between the affinity of antibody for a surface bound antigen as opposed to the affinity for a free antigen. The theory suggests a method for obtaining the affinity constants for both reactions. Extensions and applications of a mathematical model of the <u>hemolytic plaque assay</u> continued. In addition, work was begun on the development of equations to be used in the quantitation of <u>nonisotopic agglutination assays</u> , which can be used as substitutes to radio immunoassay.		

Project Description:

Objectives: To develop a simple, fast, widely available method for obtaining quantitative physical chemical information for complex reaction systems. To develop quantitative methods for obtaining kinetic and thermodynamic information on antigen antibody reactions at a single cell level. To develop methods for increasing assay reliability and precision.

Methods employed: Mathematical models; mathematical analyses of data.

Major findings: The chromatography theory is still in its early stages of development, but the equations derived have been applied by Dr. John Imman, NIAID to determine the equilibrium constant for an anti TNP Myeloma. He obtains a value within 5% of the accepted value that had previously been determined by dialysis. Work on applications of the plaque assay, especially as a method for analysis of anti-idotypic antibodies, continued but at a slow pace. Most effort was directed toward developing a quantitative theory of non isotopic aggregation assays whose use as replacements for radio immunassays is continuing to increase. Equations were derived that will allow assay optimization including an analysis of error structure of the system.

Significance to Biomedical Research and the Program of the Institute: The chromatography project is the basic component of projects related to the physical chemistry of cellular recognition and regulation. It will provide the thermodynamic and kinetic data required to develop a quantitative understanding of cellular regulation. The work on plaques is intimately related to the project on B cell regulation. It makes possible a method for studying cellular selection. It also provides a potentially new and valuable method for quantitating under appropriate conditions, the antiidiotypic antibody response. Immunoassays are important, for among other things, the detection and quantitation of low concentrations of ligands in the serum. Aside from the ability to detect abnormally low or high values of hormones that are normally present, they are potentially useful in the detection of tumor associated antigens.

Proposed Course: The reliability of column chromatography for obtaining various types of physical chemical information depends in a complicated way on the interplay between transport velocity, various types of diffusion phenomena, and movement in and out of bead, as well as on properties intrinsic to the reaction being studied. We will develop procedures for optimizing column parameters for the determination of rate constants, and information on heterogeneity in equilibrium constants. We will develop an understanding of the theoretical limits of the methods, and the techniques will be implemented experimentally by Drs. Imman and Chaiken for different systems. Simulation of the system in the absence and presence of errors in order to develop methods for optimization under a variety of experimental conditions. The mathematical analysis of augmentable plaques as a quantitative method for monitoring the dynamics of suppressive complexes in the immune response, will be implemented.

Publications

DeLisi, C. and Hethcote, H.: A theory of column chromatography for sequential reactions in heterogeneous non equilibrium systems: Application to antigen antibody reactions. Analytical Chemistry Symposia Series. Amsterdam, Elsevier. 1981

DeLisi, C. and Hiernaux, J.: Mathematical analysis of augmentable plaque forming cells: a quantitative method for monitoring auto antiideotypic antibody. In DeLisi, C., and Hiernaux, J. (Eds.) Regulatory Implications of Oscillatory Dynamics in the Immune Response. Florida, CRC Press, 1981. In press.

Hethcote, H., and DeLisi, C.: A non equilibrium model of liquid column chromatography I. Exact expressions for elution profile moments. J. Chromatog. 240: 209-281, 1982.

DeLisi, C., and Hethcote, H.: A non equilibrium model of liquid column chromatography. I Explicit solutions for the profile and effects on non ideal mass transfer rates. J. Chromatog. 240: 283-295, 1982.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08331-06 LTB																								
PERIOD COVERED <div style="text-align: center;">October 1, 1981 to September 30, 1982</div>																										
TITLE OF PROJECT (80 characters or less)  <div style="text-align: center;">Glucose-Insulin Kinetics. Modeling of Carbohydrate Metabolism</div>																										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																										
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">Mones Berman, Ph.D.</td> <td style="width: 33%;">Chief</td> <td style="width: 33%;">LMB NCI</td> </tr> <tr> <td rowspan="5">OTHERS:</td> <td>Meryl Wastney, Ph.D.</td> <td>Visiting Fellow</td> <td>LMB NCI</td> </tr> <tr> <td>David Covell, Ph.D.</td> <td>Staff Fellow</td> <td>LMB NCI</td> </tr> <tr> <td>Susan E.H. Hall, Ph.D.</td> <td>Ottawa Civic Hospital, Canada</td> <td></td> </tr> <tr> <td>Jesse Roth, M.D.</td> <td>Chief</td> <td>DB NIAMDD</td> </tr> <tr> <td>Reubin Andres, M.D.</td> <td>Gerontology Research Center, Baltimore</td> <td></td> </tr> <tr> <td></td> <td>Jordan Tobin, M.D.</td> <td>Gerontology Research Center, Baltimore</td> <td></td> </tr> </table>			PI:	Mones Berman, Ph.D.	Chief	LMB NCI	OTHERS:	Meryl Wastney, Ph.D.	Visiting Fellow	LMB NCI	David Covell, Ph.D.	Staff Fellow	LMB NCI	Susan E.H. Hall, Ph.D.	Ottawa Civic Hospital, Canada		Jesse Roth, M.D.	Chief	DB NIAMDD	Reubin Andres, M.D.	Gerontology Research Center, Baltimore			Jordan Tobin, M.D.	Gerontology Research Center, Baltimore	
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COOPERATING UNITS (if any)  Clinical Physiology Branch, GRC, NIA, Baltimore; Diabetes Branch, NIAMDD; Dept. of Physiology, Univ. of Ottawa; Div. of Metabolism, Ottawa Civic Hospital, Ontario LAB/BRANCH <div style="text-align: center;">Laboratory of Mathematical Biology</div>																										
SECTION  <div style="text-align: center;">Office of the Chief</div>																										
INSTITUTE AND LOCATION  <div style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</div>																										
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																										
SUMMARY OF WORK (200 words or less - underline keywords)  <p> <u>Kinetic models</u> of the <u>glucose regulatory systems</u> are being developed for man and other animals in collaboration with several experimental groups. These include models of the distribution, metabolism and receptor binding of <u>insulin</u>, using data from both radioiodinated and native insulins, and the production and utilization of glucose as elucidated by tracer studies with labeled glucose, alanine and lactate. Models of the role of insulin in the control of <u>glucose utilization</u> and <u>production</u> are also being studied. The kinetics of ketones in man have also been studied and modelled.         </p>																										

**Project Description:**

Objectives: To develop a qualitative and quantitative understanding of glucose homeostasis including the role which various hormones play in the production and utilization of glucose and the role which glucose plays in the secretion of insulin in normal and perturbed metabolic states. To study the various precursors of glucose and other substrates that relate to glucose utilization.

Major Findings: The development of a glucose-insulin model is a long range process of integration of various subsystems. In the present phase the kinetics of the various glucose precursors (alanine, lactate, pyruvate, etc) have been integrated into a general model with glucose to permit further development and testing of the total model and its parts. Data from the literature were imposed as additional constraints on the model resulting in a more fully defined and compatible model (Wastney). Newer studies on insulin secretion obtained from the NIA group (Drs. Andres, Tobin) have been examined with our previously developed model for insulin secretion, and additional improvements in the model were incorporated (Covell). The total glucose-insulin feedback loop was examined for its potential use as a diagnostic and therapeutic tool (Covell).

An additional component in the study of intermediate metabolism was introduced by modeling the kinetics of ketone bodies in man (Wastney, Hall). Some anomalies have been identified in the analysis of ketone kinetics using conventional methods (areas under specific activity curves, etc), and a model is being developed to deal with these. It is proposed that either there is a labeled moiety other than aceto-acetate and beta-hydroxybutyrate in plasma to account for the tracer and tracee data or else there are experimental artifacts that need to be resolved.

Significance to Biomedical Research and the Program of the Institute: Carefully tested models provide predictions as to how a complicated regulatory system will respond to a given perturbation. The clinically or experimentally observed responses seen in various altered metabolic states can thus be analyzed using such models to gain insight into the basic perturbations which have occurred in these states. Obesity, diabetes, aging and the cachexia of advanced cancer are examples of metabolic conditions in which glucose homeostasis is perturbed and can thus be better understood by use of these models.

Proposed Course: 1) To continue the study of the regulatory behavior of the glucose-insulin system in an attempt to identify the primary perturbations in the obese, diabetic and aged subjects for which data are available. 2) To extend the insulin model to account for differences between studies using radioiodinated and native insulin, and to include new information on the binding and degradation of insulin. 3) To study the biological effects of insulin action. 4) To identify the physiological process reflected in the models.

Publication:

Goebel, R., Berman, M., and Foster, D.: A mathematical model for the distribution of isotopic carbon atoms through the tricarboxylic acid cycle. Fed. Proc. 41: 96-103, 1982.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)		U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT		PROJECT NUMBER  Z01 CB 08335-06 LTB	
PERIOD COVERED October 1, 1981 to September 30, 1982					
TITLE OF PROJECT (80 characters or less) "Targeting" Liposomes for Selective Interaction with Specific Cells and Tissues					
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT					
PI:		John N. Weinstein, M.D., Ph.D.		Investigator LTB NCI	
OTHERS		Robert P. Blumenthal, Ph.D.		Chief, Membrane Structure and Function Section LTB NCI	
COOPERATING UNITS (if any) S.O. Sharrow, J. Wunderlich, L. Leserman, Centre d'Immunologie, France R. Magin, U. Illinois Champaign-Urbana					
LAB/BRANCH Laboratory of Mathematical Biology					
SECTION Office of the Chief					
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205					
TOTAL MANYEARS:		PROFESSIONAL:		OTHER:	
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS					
SUMMARY OF WORK (200 words or less - underline keywords) When hapten-modified liposomes were "targeted" to <u>myeloma</u> cells or to human <u>lymphocytes</u> , they bound in large numbers to the cell membranes, but their contents were not internalized. However, when liposomes opsonized with IgG were presented to murine P388D <sub>1</sub> cells, they were readily endocytosed. Uptake was specifically mediated by the <u>Fc receptor</u> on the cell. If <u>methotrexate</u> (MTX) was encapsulated in the liposomes, it escaped from the phagolysosomal apparatus to reach a cytoplasmic target and affect the physiology of the cell. We have developed a method for covalently attaching antibody and other ligands to liposomes. We have designed "temperature-sensitive" liposomes that release an entrapped <u>drug</u> locally at temperatures obtainable by mild <u>hyperthermia</u> , for example in the treatment of tumors. In the presence of serum the ratio of drug release at 43° to that at 37° can be made greater than 100:1. We find that (i) such liposomes deliver at least 14 times as much MTX to heated <u>murine tumors</u> as to unheated control tumors, (ii) the drug reaches its target enzyme in the tumor cell cytoplasm, and (iii) tumor growth can thus be delayed. With large unilamellar liposomes, serum leads to even faster and more useful release at T <sub>c</sub> .					

Project Description:

Objectives: To investigate the use of liposomes both in cell biology and in clinical therapy. Within this broad context,

(1) To explore the use of antigen-antibody interactions to achieve selective association of liposomes with particular cell types;

(2) To develop synergistic interactions between "temperature-sensitive" liposomal drug carriers and hyperthermic treatment;

(3) To identify the mechanisms of spontaneous, serum-induced, and osmotically-induced release of solutes from liposomes.

Non-standard Methods Employed: (a) Preparation of liposomes, by bath and probe sonication. (b) Investigation of liposome-cell interactions using the fluorescence-activated cell sorter. (c) Dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory for the purpose. (d) Measurements of release of solute from liposomes by "fluorescence self-quenching". (e) Determination of the mobility of vesicles coupled to cells, using fluorescence photobleaching. (f) Microwave heating of tumors.

Major Findings:

Objective 1: (a) Bivalent antibody selectively binds DNP-bearing liposomes to TNP-bearing lymphocytes, but the binding does not increase delivery of liposome contents to the cell interior (beyond the amount internalized spontaneously). (b) Endogenous surface IgA on cells of the murine myeloma MOPC 315 can bind liposomes bearing the appropriate hapten (DNP) to the cell surface. However, as in the lymphocyte system, binding does not increase delivery to the cytoplasm. (c) Lipid vesicles containing fluorescent molecules are potentially useful as markers for sparse or low-affinity cell-surface determinants. They can be made to contain many fluorophore molecules, thus amplifying the signal. They give very low non-specific background since the fluorophore is sequestered, and appropriate lipids are not very "sticky". (d) Liposomes are removed from the circulation of a mouse much faster if the mouse carries a myeloma secreting antibody to a hapten on the liposome. (e) Antibody-mediated binding of methotrexate-containing vesicles to MOPC 315 and TEPC 15 myeloma cells did not lead to entry of drug into the cells and inhibition of their metabolism. (f) IgG opsonized DNP-vesicles are bound in large numbers to Fc receptor-bearing cells (murine P388D<sub>1</sub>), and are then endocytosed. Liposome-encapsulated MTX then escapes the phagolysosomal system to reach a cytoplasmic target (dihydrofolate reductase) and affect the physiology of the cell. (g) IgG, protein A, avidin, and other ligands can be coupled efficiently to liposomes by use of the heterobifunctional cross-linking agent N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate. This method of coupling results in only minimal aggregation and little leakage of vesicle contents. Liposomes bearing covalently coupled mouse monoclonal antibody against human  $\gamma_2$ -microglobulin bind specifically to human cells, but not to mouse cells.

Objective 2: (a) Small unilamellar vesicles of 3:1 dipalmitoylphosphatidylcholine - distearoylphosphatidylcholine release their contents very slowly at 37°C, much faster at 41 - 46°C. Such "heat-labile" liposomes appear useful in achieving high drug concentrations selectively in local areas of hyperthermic treatment, for example, in the treatment of tumors. (b) The temperature-dependence of the release



can be enhanced by increasing the rate of temperature change, by using multilamellar vesicles in place of the unilamellar ones, and most markedly, by the presence of serum in the medium. Ratios of greater than 100:1 can be obtained for release (of a fluorescent marker) at 43 and at 37°. (c) The effect of serum is largely due to interaction of serum lipoproteins (VLDL, IDL, LDL, and HDL) with the liposomes. (d) Four times as much methotrexate was delivered to subcutaneous Lewis lung tumors heated to 42° as to unheated controls in the same animals at 36°; with L1210 tumor the ratio was 14:1. (e) Growth of the L1210 tumors was delayed by such treatment more than could be accounted for by the separate effects of heating and liposomal drug administered separately. (f) Large unilamellar vesicles are stable below T<sub>c</sub> but release their contents within a few seconds upon passage through T<sub>c</sub> with serum.

Objective 3: (a) Release of carboxyfluorescein from small unilamellar vesicles takes place by "leakage", not by an all-or-nothing "rupture" of the vesicle. (b) The rate constant for leakage increases in inverse proportion to the hydrogen ion concentration of the medium. (c) Liposomes of dioleoyl lecithin leak their contents and form structures with a characteristic appearance in negative-staining electron microscopy when allowed to interact with HDL or LDL. The interaction is faster and more pronounced with isolated HDL apolipoprotein than with the whole lipoprotein particle. (d) Liposomes bearing the DNP-hapten can be made to release carboxyfluorescein in the presence of complement and IgG anti-TNP. Fluorescence self-quenching provides the most sensitive technique available for continuously monitoring such processes. (e) Cholesterol-containing liposomes can pass intact, and without releasing much of their contents, from the peritoneum to the bloodstream of a mouse. This finding is potentially important for possible clinical instillations of liposomes I.P.

Significance to Biomedical Research and the Program of the Institute: The three objectives listed clearly relate to the possibilities of using liposomes in tumor therapy. A major barrier to such efforts has been the difficulty of directing liposomes to particular cells or anatomical sites. The studies of antibody-mediated "targeting" suggest a way to achieve selectivity but also demonstrate an additional problem: how to get the liposome and its contents into the cell after binding. The studies of synergism between liposomes and hyperthermia indicate a new way to achieve selective delivery. Use of the liposome as a hapten-carrier for analysis and sorting of cell populations may be useful in a number of areas of cellular and tumor immunology. The covalent attachment of immunoglobulin and ligands to liposomes will make possible a wide range of cell biological studies.

Proposed Course: Each of the objectives specified earlier will be pursued. In the case of the hyperthermia system, emphasis has now shifted from in vitro to in vivo approaches and also to theoretical analysis of the pharmacokinetics. Concurrently, optimal drug choice and liposome type are being sought. Studies of antibody-mediated "targeting" have now been extended to include assays of the physiological effects of delivered drugs in vitro, as well as the distribution of fluorescence markers.



Publications:

Sharrow, T.M., Sharrow, S.O., Weinstein, J.N., Ferguson, W.J., and Sternfeld., M.: RITC; A new dye for two-color immunofluorescence. *J. Histochem. and Cytology*. In press.

Leserman, L.D., Weinstein, J.N., Moore, J.J., and Terry, W.D.: Specific interaction of myeloma tumor cells with hapten-bearing liposomes containing methotrexate and carboxyfluorescein. *Cancer Research*. 40: 4768-4774, 1980.

Weinstein, J.N., Klausner, R.D., Innerarity, T.L., Ralston, E., and Blumenthal, R.: "Phase transition release" (PTR), a new approach to the interaction of proteins with lipid vesicles: Application to lipoproteins. *Biochim. Biophys. Acta*. 647: 270-284, 1981.

Innerarity, R.E., Innerarity, T.L., Weinstein, J.N., and Mahley, R.W.: Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages *in vitro* by fluorescence microscopy. *Atherosclerosis*. 1: 177-185, 1981.

Feinendegen, M.B., Muhlensiepen, H., Porschen, W., Weinstein, J.N., and Feinendegen, R.E.: Selective delivery of liposome encapsulated cis-dichlorodiammineplatinum (II) by heat: Influence on tumor drug uptake and growth. *Cancer Research*. 41: 602-607, 1981.

Weinstein, J.N., Leserman, L.D., Hankart, P.a., and Blumenthal, R.: Targeting of liposomes. In Gregorladis, G., and Papahadjopoulos, D., (Eds.): *Targeting of Drugs*. N.Y. Plenum, in press.

Ralston, E., Hjelmeland, L.M., Klausner, R.D., Weinstein, J.N., and Blumenthal, R.: Carboxyfluorescein as a probe for liposome-cell interactions: Effect of impurities, and purification of the dye. *Biochim. Biophys. Acta*. 649: 133-137, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08340-04 LTB
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PERIOD COVERED

October 1, 1981 to September 30, 1982

TITLE OF PROJECT (80 characters or less)

Physical Chemistry of Antibody Effector Functions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Charles DeLisi, Ph.D. Chief, Theoretical Immunology Section LMB NCI

COOPERATING UNITS (if any) Dr. Ruben Siraganian, Clinical Immunology Section, NIDR; Prof. George Barisás, Biochemistry Dept. Univ. of St. Louis Med. School; Alan Perelson, Ph.D., Los Alamos National Lab., Los Alamos, NM; Dr. David Segal, Immunology Branch, DCBD, NCI; Dr. Steve Dower, Immunology Branch, DCBD, NCI

LAB/BRANCH

Laboratory of Mathematical Biology

SECTION

Theoretical Immunology Section

INSTITUTE AND LOCATION

NCI, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.1

PROFESSIONAL:

0.1

OTHER:

0

CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS

☒ (b) HUMAN TISSUES

☐ (c) NEITHER

☐ (a1) MINORS ☐ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords) Work on the basophil system was continued with the development of a new theory of the kinetics of activation and specific desensitization of cells from allergic and immunized individuals. The theory has been applied to the analysis of a wide variety of data. We also developed new methods, based on measurements of the kinetics of cell activation, for determining whether or not the descending limb of biphasic dose response curve falls because of insufficient cross-linking. We studied equilibrium and kinetic properties of IgG diatomers of defined size interacting with Fc receptors on a macrophage-like cell line. The results of the equilibrium studies provided the first experimental evidence in support of our predictions that receptor cross-linking can lead to non linear Scatchard plots. In addition, the data suggested two different types of binding sites for dimeric and trimeric oligomers, but only a single type for monomers. The maximum affinity enhancements -- 200 for dimeric relative to monomeric and 2.5 for trimeric relative to dimeric, indicate considerable strain or large differences in the entropic parts of the equilibrium constants for solution phase as apposed to cell surface reactions. The dissociation kinetics of dimer and trimer were biphasic and the rate of dissociation was accelerated by high concentrations of monomer.

## Project Description:

**Objectives:** (1) To develop an understanding of the relationship between cell surface events and the biochemical pathway which a cell follows. In the basophil system this means sorting out the parameter domains corresponding to specific desensitization, non specific desensitization, histamine secretion, and inactivity. (2) To develop a detailed understanding of Fc receptor redistributions upon the binding antigen-antibody complexes. (3) To determine and study the relation between cluster size and distribution and cellular activity. (4) To develop an understanding of the relationship between the thermodynamics and kinetics of ligand cell-bound receptor interactions, and the requirements for lymphocyte activation.

**Methods Employed:** Mathematical modeling; mathematical analysis of experimental data.

**Major Findings:** Basophils sensitized to penicillin degranulate and release histamine when incubated with multivalent penicillin derivatives but not with monovalent hapten. The dose-response curve is biphasic with maximal release at a concentration of dimeric hapten of about 1 nM. The characteristics of the response generated using mixtures of monovalent and divalent derivatives, as well as a variety of other evidence, suggest that the rise and fall in the dose-response curve reflects the rise and fall in the concentration of receptors cross-linked by the multivalent hapten.

Extracellular  $\text{Ca}^{2+}$  is required for histamine release and cells may be desensitized to different degrees by incubation with various concentrations of ligand in the absence of  $\text{Ca}^{2+}$ . Washing and rechallenging cells with an optimal dose of ligand and  $\text{Ca}^{2+}$  indicates that the dose dependence of desensitization is also biphasic and is most pronounced at a ligand concentration which ordinarily stimulates maximal release. The implication is that cross-linking, which, for the thermodynamic parameters characteristics of this system, will lead primarily to dimers and trimers, initiates signals for both degranulation and desensitization.

Kinetic studies reveal release curves which are sigmoidal, having delays in the onset of release that vary with ligand concentration. The magnitude of the delay is a biphasic function of concentration with a maximum at approximately the same concentration as the peak in the dose-response curve. The results are interpreted in terms of a model in which cross-linked receptors are converted to an active, unstable intermediate which facilitates an increase in cytoplasmic  $\text{Ca}^{2+}$ , but which decays spontaneously into an inactive product. Dependence of histamine release on the concentration of the intermediate is nonlinear, suggesting either a positive feedback loop stabilizing the intermediate or the interaction of several aggregates. A fit of a simple mathematical formulation of the model indicates that it qualitatively and quantitatively explains the dose-response, desensitization, and release patterns.

Scatchard plots for IgG oligomers are convex to the origin. The result provides the first direct experimental evidence that receptor clustering leads to such plots. The rate of dissociation of labeled ligand is faster in the presence of cold ligand than in its absence. The result again provides the first direct experimental evidence that receptor clustering can cause such acceleration. Both effects are widely observed in cell systems, and they have

previously been attributed to negative binding cooperativity caused by conformational changes in the receptor. The results therefore may require a reassessment of the interpretation of a wide variety of data. The method in principle should be applicable to any cell that responds via a receptor clustering mechanism. For such systems it is important to know whether failure of the cell to respond at high ligand concentrations is simply the result of inability to cross-link (because all receptor sites are saturated) or the result of some desensitization signal induced by large aggregates. We have currently identified AgE and human basophils as a system in which a desensitization signal prevents release at high concentrations.

Significance to Biomedical Research and the Program of the Institute: Aside from the obvious bearing on allergic reactions, the basophil system, because it is a one ligand-one cell system which responds in minutes, permits the type of quantitative analysis which is not possible for antibody production and secretion. It is therefore a model system for studying cellular activation.

This project is related to the projects on lymphocyte activation, complement activation and insulin binding. For the first two, binding of antibodies to cells via the Fc receptor must precede biological activity. A detailed understanding of the nature of the complexes formed is a necessary component of developing an understanding of the biological processes. The problem of determining and quantitating the parameters that effect the initial states of signal transduction in basophils is an important aspect of the attempt to understand the nature of the immediate allergic reaction. More generally, however, the basophil system may serve as a model from which to build a deeper understanding of the relation between the binding of ligand to cell surface receptors and subsequent biochemical events and factors that regulate them.

Proposed Course: Experimental tests of many of the predictions are now in progress. The model will be extended to include non specific desensitization.

The theory makes predictions related to the effect of variations in the mean number of cell surface receptors which will be tested experimentally. The relation between binding and activity under various conditions will be determined and analyzed. The effect of fluidity on complement fixation will be assessed.

#### Publications:

Perelson, A., DeLisi, C., and Siraganian, R.: A method for determining whether the descending limb of biophasic dose response curve reflects insufficient cross-linking. Molec. Immunol. 19: 15, 1982.

Dower, S.K., DeLisi, C. Titus, T.A., and Segal, O.M.: The mechanisms of binding of multivalent immune complexes to Fc receptors I: Equilibrium studies. Biochemistry. 20: 6326-6334, 1981.

Dower, S.K., Titus, J.A., DeLisi, C., and Segal, D.M.: The mechanism of binding of multivalent immune complexes to Fc receptors II: Kinetics of binding. Biochemistry. 20: 6335-6340, 1981.

DeLisi, C., DelGrosso, G., and Marchetti, F.: A theory of measurement error and its implications for spatial and temporal gradient sensing during chemotaxis. Cell Biophys. In press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08341-04 LTB								
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>										
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Physical Chemical Studies of Lipid-Protein Interactions</p>										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT										
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">John N. Weinstein, M.D., Ph.D.</td> <td style="width: 33%;">Investigator</td> <td style="width: 15%;">LTB NCI</td> </tr> <tr> <td>OTHERS</td> <td>Robert P. Blumenthal, Ph.D.</td> <td>Chief, Membrane Structure &amp; Function Section</td> <td>LTB NCI</td> </tr> </table>			PI:	John N. Weinstein, M.D., Ph.D.	Investigator	LTB NCI	OTHERS	Robert P. Blumenthal, Ph.D.	Chief, Membrane Structure & Function Section	LTB NCI
PI:	John N. Weinstein, M.D., Ph.D.	Investigator	LTB NCI							
OTHERS	Robert P. Blumenthal, Ph.D.	Chief, Membrane Structure & Function Section	LTB NCI							
COOPERATING UNITS (if any) <p style="text-align: center;">T. Innerarity and R. Pitas, Univ. of California at San Francisco; Richard Klausner, LBM, NIAMDD</p>										
LAB/BRANCH <p style="text-align: center;">Laboratory of Mathematical Biology</p>										
SECTION <p style="text-align: center;">Office of the Chief</p>										
INSTITUTE AND LOCATION <p style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</p>										
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0.4	0.4	0								
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <span><input type="checkbox"/> (a) HUMAN SUBJECTS</span> <span><input checked="" type="checkbox"/> (b) HUMAN TISSUES</span> <span><input type="checkbox"/> (c) NEITHER</span> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <span><input type="checkbox"/> (a1) MINORS</span> <span><input type="checkbox"/> (a2) INTERVIEWS</span> </div>										
SUMMARY OF WORK (200 words or less - underline keywords) <p>             We have investigated the interaction of <u>lipoproteins</u> with <u>liposomes</u> to form recombinant particles. A number of lipoprotein fractions (VLDL, IDL, LDL, and HDL) all disrupt liposome structure by an essentially irreversible and quasi-stoichiometric process. In the case of HDL, the major apoprotein, A-I, recombines with dimyristoyl phosphatidyl choline vesicles at 40:1 lipid:protein to form discs approximately 100 Å in diameter and 32 Å in thickness, with protein on the rim. These structural results were obtained by a combination of <u>neutron scattering</u>, <u>electron microscopy</u>, and <u>column chromatography</u>.              With dipalmitoyl phosphatidylcholine, A-I also forms what we term "<u>vesicular recombinant</u>" particles in a process which may relate to physiological mechanisms by which proteins are assembled into membranes and lipoproteins. To study this process we have developed a technique called "<u>phase transition release</u>" (PTR) which is also being applied to study incorporation of <u>tubulin</u> into membranes.              Lipoproteins were labelled with the fluorescent lipid 3,3-dioctadecylindocarbocyanine for studies of interaction with cell surface lipoprotein receptors. The lipoproteins are also being labelled with NBD lipids for two-color fluorescence identification of cells in atherosclerotic plaques.           </p>										



Project Description:

Objectives: To investigate the interaction between liposomes and lipoprotein and between lipoproteins and cells. More specifically,

(1) To define the mechanism by which liposomes are broken down by serum components, principally the lipoproteins;

(2) To determine the relative efficacies of different purified apolipoproteins in breaking up liposomes;

(3) To develop fluorescently labelled lipoproteins and liposome-apoprotein recombinants for use in studying lipoprotein-cell interactions;

(4) To use the methods developed for objectives (1) - (3) to assess the physical chemistry of protein-lipid interaction in lipoproteins;

(5) To extend to other bilayer-protein interactions the concepts thus developed;

(6) In particular, to investigate the assembly of tubulin and actin into membranes.

Non-standard Methods Employed: (a) Preparation of liposomes, by probe and bath-sonication; (b) Dynamic measurement of leakage from liposomes as a function of temperature, using a temperature-scanning fluorescence system devised in our laboratory, i.e., "phase transition release" (PTR); (c) Determination of the mobility of lipoproteins and liposomes bound to the cells, using fluorescence-photobleaching recovery; (d) Neutron scattering studies of lipoprotein size and shape; (e) Aqueous collisional fluorescence quenching studies of lipid-protein interaction; (f) Derivatization of proteins with trinitrobenzene sulfonate; (g) labelling of lipoproteins with 3,3'-dioctadecylindocarbocyanine (diI) dye.

Major Findings: (1) Liposomes are broken down and their contents released by an interaction with serum, most rapidly at the lipid phase transition. (2) The serum effect is mostly due to low density (LDL) and high density (HDL) lipoproteins, but very low density and intermediate density lipoproteins also play a part, as also does some other, unidentified component of the serum. (3) Heparinized plasma, EDTA plasma, and serum all have the same effect. (4) At least in the case of apo-HDL the mechanism appears to be a quasi-stoichiometric, all-or-nothing breakdown of the vesicle into a small disc of lipid rimmed with protein. The process is largely complete within a second or two at the phase transition of the liposome lipid and is essentially irreversible on that time scale. (5) By neutron diffraction and electron microscopic studies the discs appear to be about 100 Å in diameter and 32 Å in thickness, with a protein rim. They probably consist of a single bilayer. (6) Cholesterol at 40 mole percent in the liposomes severely restricts the interaction with serum components, as does the formation of liposomes from lipids which are below their phase transitions at the temperature of study. (7) At molar ratios of about 2,000:1 dipalmitoyl phosphatidylcholine:Apo A-I, a stable vesicular recombinant particle (VR) is formed below the lipid phase transition temperature (T<sub>c</sub>). As the temperature is raised through T<sub>c</sub> (in PTR), a new type of recombinant (VR-T<sub>c</sub>) is formed. By physical measurements of several types, the A-I's conformation and disposition in the lipid change at T<sub>c</sub>. The protein probably becomes trans-membrane. These findings may relate to physiology processes for formation of

HDL and to the assembly of intrinsic proteins into membranes. (8) Purified tubulin interacts with vesicles at Tc to form vesicular recombinants. The interaction is accompanied by structural changes in lipid and protein. (9) HDL, LDL, and apoE-HDL can all be labelled efficiently and irreversibly with the fluorescent lipid analogue, diI. The lipoproteins are unchanged in physical properties and in specific binding to cell surface receptors. The labelled lipoproteins were acetoacetylated for studies of phagocytic and lipoprotein-specific uptake in arterial walls. (10) Studies of the interaction with bilayers of a hepatic membrane receptor for asialoglycoprotein are described in another report (Z01 CB 08343-01 LTB).

Significance to Biomedical Research and the Program of the Institute: (1) A major barrier to the effective use of liposomes as carriers in cancer chemotherapy and diagnosis has been a lack of understanding of their interactions with serum. Our studies define the interaction and indicate what type of liposomes must be used to avoid it. (2) Our studies of HDL-liposome recombinants contribute to (1) and may also be useful in delineating mechanisms of atherosclerosis. Our fluorescently labelled lipoproteins are currently being used by collaborators to study atherogenesis. (3) Breakdown of liposomes in serum is essential to the combination of liposomes with hyperthermia to achieve selective release of drugs in the area of a tumor.

Proposed Course: Lipoproteins and liposome-lipoprotein recombinants are being labelled with both red and green fluorophores for studies of their physical chemistry and interactions with cells; the requirements for a serum-stable liposomal carrier are being defined, with emphasis now on the differences among different size liposomes.

Publications:

Weinstein, J.N., Klausner, R.D., Innerarity, T.L., Ralston, E., and Blumenthal, R.: "Phase transition release" (PTR), a new approach to the interaction of proteins with lipid vesicles: application to lipoproteins. Biochim. Biophys. Acta. 647: 270-284, 1981.

Pitas, R.E., Innerarity, T.L., Weinstein, J.N., and Mahley, R.W.: Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. Atherosclerosis. 1: 177-185, 1981.

Kumar, N., Klausner, R.D., Weinstein, J.N., Blumenthal, R., and Flavin, M.: Interaction of tubulin with phospholipid vesicles II: physical changes of the protein. J. Biol. Chem. 256: 5886-5889, 1981.

Klausner, R.D., Kumar, N., Weinstein, J.N., Blumenthal, R., and Flavin, M.: Interaction of tubulin with phospholipid vesicles I: association with vesicles at the phase transition. J. Biol. Chem. 256: 5879-5885, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08342-03 LTB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Theory of Receptor-ligand Biophysics		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Charles DeLisi, Ph.D.                      Chief, Theoretical Immunology Section                      LTB NCI		
COOPERATING UNITS (if any) Dr. Alan Perelson, Theor. Div. Los Alamos National Lab., Los Alamos, NM; Prof. Frederik Wiegel, Dept. of Physics, Twente Univ. of Tech., Enschede, Netherlands; Prof. Federico Marchetti, University of Rome, Rome Italy		
LAB/BRANCH Laboratory of Mathematical Biology		
SECTION Theoretical Immunology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL, MANYEARS: 0.4	PROFESSIONAL: 0.4	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <u>Rate constants for ligands interacting with cell bound or dispersed receptors</u> have a diffusible part and an intrinsic part: the former depending on geometry, receptor distributions, and <u>diffusion coefficients</u> ; the latter on electronic redistributions. We have been focusing on the former and have obtained expres- sions for diffusion limited association and dissociation rate constants when (1) ligands bind directly and specifically to receptors that are distributed over a spherical surface; (2) ligands bind indirectly by a path that includes <u>non</u> <u>specific association</u> with the cell and <u>diffusion in the surface</u> , toward or away from a specific receptor. We have also developed a formalism that permits calculation of the complete equilibrium and rate constants for cell bound receptors, given the equilibrium or rate constants for dispersed receptors. Mathematical methods are also being developed to describe <u>aggregation</u> on a <u>two dimensional fluid surface</u> .		

Project Description:

Objective: To obtain expressions that can be used to analyze kinetic data for reactions between ligands and cell bound receptors. To obtain analytic expressions for the time evolution of the distribution function describing the growth of aggregates subsequent to the binding of ligand to laterally mobile cell surface receptors.

Major Findings: The kinetic problem for the entire distribution function, with and without loop closure (cyclic complexes) was solved exactly for the first time for a bivalent-bivalent system. Because the formulation of the problem leads to an infinite system of coupled non-linear equations, numerical solutions are not possible. The analytical results thus allow applications that were previously not possible.

The theory is being used to answer a variety of questions of biological interest. One of its applications, the analysis of ligands dissociating from cell surface receptors, predicts that accelerated dissociation by cold ligand and multi exponential decay of label, is expected even in the absence of negative cooperativity or cross-linking. The conditions under which such observations are expected have been derived, and a consideration of known rate constants indicates that they are likely to be met in a large number of cases.

Clustering of receptors is responsible for cell activation and/or desensitization in a variety of systems. Moreover, since very few clusters - perhaps three or four - are needed to induce activity in some systems the question of how cells prevent spontaneous activation arises. Equilibrium calculations based on the entropic loss in constraining receptors to be adjacent, indicate that hundreds of pairs are expected on cells having  $10^5$ - $10^6$  receptors.

The results of this project shed light on this problem. The main idea is that receptors must remain within some minimum distance of one another for some required amount of time to transduce a signal. The equations indicate that receptors will diffuse out of this required distance very rapidly, on the order of  $10^{-5}$  seconds. Thus for transduction times of the order of a millisecond, the ratio of separation time to transduction time (the central parameter) is in the range of 10-100. One can then show that the probability of spontaneous transduction can easily be as low or lower than  $10^{-30}$ . With even a weak affinity ligand, the probability changes to close to one. Thus a control mechanism based on a residence time requirement can easily lead to virtually infinite amplification of transduction.

Significance to Biomedical Research and the Program of the Institute : The methods are required to develop an understanding of the physical chemistry of the regulation of cell responsiveness at the cell surface level. Aspects of these ideas may be relevant to a wide range of biological processes including enzymic enhancement of catalysis. The results will also allow correlations to be drawn about cellular activity and the size of the aggregates which form on the cell surface, i.e., previously it was possible only to connect the mean number of crosslinked receptors with activity; now the importance of the way they are distributed in various sized aggregates can be assessed. This project is a necessary aspect of all other research projects.



Proposed Course: Additional fundamental work on estimating the accuracy with which a cell can sense its environment; applications to a variety of dose response data including lysozomal enzyme release, histamine secretion, chemo-reception and complement activation. Applications to basophils and mast cells responding to multifunctional antigens. Assesment of the role of valence and geometry in the regulation of the response.

Publications:

DeLisi, C. and Wiegel, F.: Effect of non-specific forces and finite receptor number on ligand-cell bound receptor rate constants. Proc. Nat. Acad. Sci 78: 5569-5572, 1981.

Pincus, M., DeLisi, C., and Rendell, M.: Ligand binding to multiple equivalent sites with steric hindrance. Biophys. Biochem. Acta. 675: 392-396, 1981.

DeLisi, C.: Toward a dynamical theory of membrane organization and function. In DeLisi, C., Perelson, A., and Wiegel, F. (Eds.): Physical Chemistry of Cell Surface Phenomena. Marcel Dekker. In press.

DeLisi, C., and Wiegler, F.: Rate constant enhancement by reduction in dimensionality is likely to be of little consequence in cellular systems. Amer J. Physiol. In press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08348-03 LTB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Receptor-ligand Kinetics and Function		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	Mones Berman, Ph.D.	Chief LMB NCI
Other:	David Covell, Ph.D.	Staff Fellow LMB NCI
	Jerry Gardner, M.D.	Chief DD NIAMDD
COOPERATING UNITS (if any) Digestive Diseases Br., NIAMDD		
LAB/BRANCH Laboratory of Mathematical Biology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: .5	PROFESSIONAL: .5	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Several <u>receptor</u> systems are being investigated by <u>modeling</u> the <u>hormone</u> <u>kinetics</u> , binding and function. Hormones investigated include <u>insulin</u> , <u>glucagon</u> , <u>secretoagogues</u> and others.		

Project Description:

Objectives: To develop a general, unified hormone-receptor model from the insights gained by studying the individual systems.

Major Findings: The kinetics of cellular calcium have been investigated in response to secretagogues (Chabay) and other stimulators (Covell). Modeling in this area has progressed slowly.

Significance to Biomedical Research and the Program of the Institute: The receptor systems are most important in the control of cellular processes and are thus relevant to much of the biological research conducted in this and other laboratories at NIH and elsewhere.

Proposed Course: Development of the model using various receptor systems in-vitro and in-vivo will continue.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08349-02 LTB
PERIOD COVERED <p style="text-align: center;">October 1, 1981 to September 30, 1982</p>		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Characterization of Macromolecular Flexibility</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Robert L. Jernigan, Ph.D.                      Theoretical Physical Chemist      LTB NCI  OTHERS: Sanzo Miyazawa, Ph.D.                      Visiting Fellow                      LTB NCI		
COOPERATING UNITS (if any)  <p style="text-align: center;">None</p>		
LAB/BRANCH  <p style="text-align: center;">Laboratory of Mathematical Biology</p>		
SECTION  <p style="text-align: center;">Office of the Chief</p>		
INSTITUTE AND LOCATION  <p style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS:  <p style="text-align: center;">0.2</p>	PROFESSIONAL:  <p style="text-align: center;">0.2</p>	OTHER:  <p style="text-align: center;">0</p>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Helical biopolymers</u> are locally <u>rigid</u>, but very high molecular weight materials can display flexibility approaching random coil behavior. The origin of this flexibility is not understood. In the case of double strand helical DNA, two origins for this flexibility have been proposed: 1) small <u>fluctuations</u> in many of the <u>rotational angles</u> which yield conformations with <u>smoothly bending trajectories</u> or 2) sharper bends whose locations depend on specific sequences. We have considered the first of these cases and have developed methods to perform averages over such conformations. Comparisons with experimentally measured persistence lengths permit determination of the average amount of torsion about backbone bonds. In the case of double strand DNA, these values are consistent with a uniformly twisting chain with mean deviations of only a 2-5 degrees. This range of fluctuations appears to be consistent with usual torsional potentials. It is expected that this flexibility would be manifested at lower molecular weights in Kerr constant measurements than for quantities such as radius of gyration.         </p>		

Objectives: To determine the importance of local torsional angle variations to the flexibility manifested by helical biopolymers.

Methods Employed: New statistical mechanical methods of introducing averages over torsional angle fluctuations of various physical quantities, such as chain dimensions and dipole moments. These local rotational fluctuations are assumed to be independent of one another.

Major Findings: Methods can be applied to torsional potentials of any characteristic foldedness and any barrier height. The average of each geometric quantity is represented by the helical value reduced by a factor that depends on the barrier height and rotational symmetry. The effectiveness of a specified fluctuation in introducing flexibility increases upon passing to higher moments. Thus experimental methods such as Kerr constant determinations would be expected to show evidence of this flexibility for smaller molecules than methods such as radius of gyration determination from light scattering. Experimental persistence length values are consistent with a root-mean-square angular fluctuation of 2-5 degrees for each backbone bond of double strand helical DNA.

Significance to Biomedical Research and the Program of the Institute: Basic understanding of the flexibility of biological helices is needed for a better understanding of the role of molecular conformations in molecular biological processes such as translation.

Proposed Course: Applications to other classes of biological helical macromolecules are anticipated, such as polypeptides and polysaccharides. Comparisons with experimental persistence lengths should provide validation or refutation of this viewpoint of flexibility. Calculations of various physical properties can serve to indicate the relative virtues of various methods in detecting such limited flexibility.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 CB 08353-02 LTB
PERIOD COVERED <div style="text-align: center;">October 1, 1981 to September 30, 1982</div>		
TITLE OF PROJECT (80 characters or less) <div style="text-align: center;">Perpetuation and Eradication of Viruses in Large Populations</div>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <span>PI: Ira B. Schwartz, Ph.D.</span> <span>Staff Fellow</span> <span>LTB NCI</span> </div>		
COOPERATING UNITS (if any) <div style="text-align: center;">None</div>		
LAB/BRANCH <div style="text-align: center;">Laboratory of Mathematical Biology</div>		
SECTION <div style="text-align: center;">Office of the Chief</div>		
INSTITUTE AND LOCATION <div style="text-align: center;">NCI, NIH, Bethesda, Maryland 20205</div>		
TOTAL MANYEARS: <div style="text-align: center;">0.5</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-around; align-items: flex-start;"> <div style="text-align: center;"> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div style="text-align: center;"> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div style="text-align: center;"> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-around; align-items: flex-start; margin-top: 5px;"> <div style="text-align: center;"> <input type="checkbox"/> (a1) MINORS         </div> <div style="text-align: center;"> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) We continue to examine the role of <u>seasonal variation in transmissibility</u> of <u>epidemic models</u> . In particular, we have developed a method for determining the rate of spread of an infection due to a <u>virus</u> using various mathematical models. Non-equilibrium situations, such as <u>measles</u> outbreaks, are considered.		



Project Description:

Objectives: We attempt to find the underlying mechanisms of seasonality in viruses that sustain undamped recurrent outbreaks of viral infections. In addition we are developing efficient software for solving numerically a general class of epidemic models. The overall objective is to extend the model to include age dependency and rates of spread of infection.

Methods Employed: Computer modelling; mathematical analysis; numerical analysis.

Major Findings: It is shown that for human virus infections such as measles, poliomyelitis and hepatitis B, seasonality plays a major role in the perpetuation of diseases. Extensive computer simulations demonstrate in the case of measles that there exist many periodic oscillations which range from small to very large fluctuations in the number of infectives. The existence of large fluctuating periodic oscillations leads to short terms of irregular oscillations in the number of cases of disease as is reported in Baltimore in the pre-vaccine era from 1928-1950. In the case of rubella, larger term outbreaks can be modelled within our simple model.

Significance for Biomedical Research and the Program of the Institute: Eradication is the converse of perpetuation and represents the ultimate methods for control of an infectious disease. To determine the potential for eradication, it is necessary first to understand the requirements for perpetuation. Thus, the subject is highly relevant to practical goals in public health and preventive medicine.

Publications:

Schwartz, I.B.: Estimating regions existence of unstable periodic orbits using computer-based techniques. SIAM Journal on Numerical Analysis. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08354-02 LTB
PERIOD COVERED      October 1 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Enzyme-Substrate Complexes		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Matthew R. Pincus, M.D., Ph.D.      Expert, Theoretical Chemist      LTB NCI		
OTHER      Sandra Smith-Gill, Ph.D.      IPA      LCB NCI		
COOPERATING UNITS (if any)  Professor Harold Scheraga, Cornell University, Ithaca, NY		
LAB/BRANCH      Laboratory of Mathematical Biology		
SECTION      Office of the Chief		
INSTITUTE AND LOCATION      NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:      0.25	PROFESSIONAL:      0.25	OTHER:
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>It is highly desirable to understand how enzymes and proteins in general bind to only specific ligands. Realization of this objective leads directly to our being able to design drugs that can bind to different receptors and enzymes with pre-determined affinities. We have been able to predict the structure of enzyme-substrate complexes using <u>conformational energy</u> calculations. We have developed a <u>global search technique</u> that allows us to compute all of the allowed binding modes for substrates at the active sites of enzymes. The strategy is applied to <u>lysozyme</u> where it is shown that we have been able to compute the structures of enzyme-substrate complexes of this enzyme that are in excellent agreement with both X-ray crystallographic and solution studies.</p>		

**Project Description:**

**Objectives:** To calculate the three-dimensional structures of enzyme-substrate complexes using conformational energy calculations; to test predicted structures experimentally, and to determine the critical interactions involved in the best structures. Applications are to lysozyme which shows strong specificity for its substrates, and which is a simple enzyme for experimental study. The ultimate object is to predict the structures of molecules that bind with specific affinities to drug receptors.

**Methods Employed:** Conformational energy calculations are used to compute all allowed conformations for the isolated substrate and the enzyme-substrate complex. Multi-dimensional phase space searches are employed to determine allowed binding regions for simpler substrates. The structures of all complexes determined in this step are then subjected to energy minimization, allowing for all internal and external degree of freedom for the substrate and internal degrees of freedom for the enzyme. We have recently predicted the structure of an enzyme-hexasaccharide complex. We have tested this calculated binding mode by using monoclonal antibodies directed against the specific binding site.

**Major Findings:** We have calculated unique, lowest energy structures for a variety of 1,4-linked N-acetylglucosamine polymers bound to the active site of the enzyme. These structures agree quite well with experimentally determined structures on the basis of X-ray crystallography. We have computed new binding sites not previously thought to be involved in the binding of saccharides to the active site of the enzyme; in particular, a different D site from the one proposed on the basis of crystallographic model-building and a new F site on the side of the cleft opposite Arg 114 (as proposed on the basis of model building), involving such residues as Arg 45, Asn 46, and Thr 47. The former prediction has been fully verified by X-ray crystallography. We are currently testing the latter calculation in the following novel way. Recently, a monoclonal antibody has been raised to lysozyme that binds quite specifically to our calculated F site, i.e., to residues 45, 46, and 47. We have now shown that the specific monoclonal antibody directed against this site is displaced by substrates that bind to the F site but not by inhibitors that do not bind to this site. In addition, we have shown that the dye, Biebrich scarlet, known to bind to the F site, displaces the antibody. We are now performing comparative studies of the binding of hexa-(GlcNAc) to hen and pheasant lysozyme. The latter enzyme has a drastically different right side sequence but an identical left-side sequence.

We have further calculated the structures of copolymers of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), which are the natural cell wall substrates for this enzyme. We have calculated that specific interactions between the lactic acid side chain in site F make the alternating copolymer the best substrate for the enzyme, in agreement with solution studies.

**Significance to Biomedical Research and the Program of the Institute:** Since our calculations have been successful in predicting structures of enzyme-substrate complexes, we can now attempt to determine which molecules would offer optimal interactions and serve as either substrates or inhibitors. Such studies can suggest molecules that might act as effective drugs.

Publications:

Pincus, M.R., and Scheraga, H.A.: Theoretical calculations on enzyme-substrate complexes: The basis of molecular recognition and catalysis. Accounts of Chemical Research. 14: 299-306, 1981.

Pincus, M.R., and Scheraga, H.A.: Prediction of the three dimensional structures of complexes of lysozyme with cell wall substrates. Biochemistry. 20: 3960-3965, 1981.

Scheraga, H.A., Pincus, M.R., and Burke, K.E.: Calculations of structures of enzyme-substrate complexes. In the Eleventh Hoechst Workshop Conference on Structures of Complexes between Biopolymers and Low Molecular Weight Molecules, October, 1981. To Be Published in Book Form.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08357-01 LTB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less)  Cell Interactions		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  P.I: Charles DeLisi, Ph.D. Chief, Theoretical Immunology Section LTB NCI		
COOPERATING UNITS (if any)  Dr. Jacques Hiernaux, LTB		
LAB/BRANCH Laboratory of Mathematical Biology		
SECTION Theoretical Immunology Section		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.2	PROFESSIONAL: 0.2	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  The number of <u>antibody secretion cells</u> in normal and nude mice was studied as a function of <u>time</u> after a single injection of either <u>bacterial levan</u> or <u>lipopolysaccharide</u> . The number of cells producing antibodies against the dominant anti BL <u>idiotype</u> was also studied.		



## Project Description:

Objective: To elucidate the mechanism(s) regulating the maintenance phase of the immune response.

Major Findings: The immune response to a single injection of bacterial levan (BL) or lipopolysaccharide (LPS) persists for months, the number of antibody producing cells oscillating synchronously in some cases and asynchronously in others. The pattern occurs in both euthymic and athymic BALB/c suggesting that auto antiidiotypic antibodies and T-cells do not play a significant role in their generation. Moreover, we found no relation between the suppressive property of auto antiidiotypic antibody (in normal mice) and either the induction or maintenance of tolerance to BL.

Significance to Biomedical Research and the Program of the Institute: The mechanisms responsible for long term maintenance are of major importance to the understanding of a number of disease related problems; e.g. allergy, arthritis, graft rejection. Since attempts to modulate the response in these instances can occur weeks or even years after induction, understanding the regulation of the maintenance phase is crucial for successful intervention.

Proposed Course: The simplest qualitative explanation for the data is based on a simple antibody mediated feedback loop which we had proposed earlier. Our most recent data, however, indicate that this idea is quantitatively inadequate. We will be developing more sophisticated models taking account of circulatory patterns and possible B cell interactions.

Publications:

Hiernaux, J., Chang, J., Baker, P., and DeLisi, C.: Lack of involvement of auto-antiidiotypic antibody in the regulation of oscillations and tolerance in the antibody response to levan. Cellular Immunol. 67: 334-345, 1982.

Hiernaux, J., Baker, P., DeLisi, C., and Rudback, A.: Modulation of the immune response to lipopolysaccharide. J. Immunol. 128: 1054- , 1982.

DeLisi, C., and Hiernaux, J. (Eds.): Regulatory Implications of Oscillatory Immune Response Dynamics. CRC Publishing Co. Vols. 1 and 2. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)		U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF INTRAMURAL RESEARCH PROJECT</b>		PROJECT NUMBER  Z01 CB 08358-01 LTB	
PERIOD COVERED December 1, 1981 to June 26, 1982					
TITLE OF PROJECT (80 characters or less)  Epidemic Cycles					
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT					
PI: Joan L. Aron, Ph.D.		Staff Fellow		LTB NCI	
OTHERS: Ira B. Schwartz, Ph.D.		Staff Fellow		LTB NCI	
COOPERATING UNITS (if any) James A. Yorke, Ph.D., Institute of Physical Science and Technology, Univ. of Maryland, College Park, MD					
LAB/BRANCH		Laboratory of Mathematical Biology			
SECTION		Theoretical Immunology Section			
INSTITUTE AND LOCATION		NCI, NIH, Bethesda, Maryland 20205			
TOTAL MANYEARS:		PROFESSIONAL:		OTHER:	
0.5		0.5		0	
CHECK APPROPRIATE BOX(ES)					
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER					
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS					
SUMMARY OF WORK (200 words or less - underline keywords) <u>Oscillations exhibited by nonlinear systems of differential equations</u> are qualitatively different when they are forced by an external oscillation applied to the system. Although some solutions of the forced system have, not surprisingly, the same period as the forcing term, there exist other solutions whose period is some integral multiple of the forcing period. Numerical computer techniques were implemented to follow the periodic orbits and to calculate their period and stability. The methods were applied to an <u>epidemic model</u> (SEIR) with birth and death rates added to insure population turnover and a steady supply of new susceptibles. The external oscillation was put into the transmission term, corresponding here to seasonal changes. The major result is that it is relatively easy to find stable periodic solutions whose periods are double that of the forcing term corresponding to some measles epidemics. Solutions with longer periods generally have much greater amplitudes. Consequently, observed non-biennial cycles of epidemics, such as those of rubella, probably are fundamentally different.					

Project Description:

Objectives: To understand the driving force behind different temporal patterns of recurrent epidemics.

Major Findings: Seasonal changes in transmission, which had already been shown to cause biennial epidemics, cannot be simply related to other patterns of recurrent epidemics with longer periods. Structurally different models are probably needed to explain other periods of recurrence.

Significance to Biomedical Research and the Program of the Institute: Understanding periodic recurrences of epidemics may lead to a better understanding of the attempts to control disease and better prediction of outbreaks.

Proposed Course: Consider alternative epidemic models of non-biennial cycles of epidemics, based on available data.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  201 CB 08359-01 LTB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Monoclonal Antibodies In Vivo for Diagnosis and Therapy of Tumors		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: John N. Weinstein, M.D., Ph.D.	Investigator	LTB NCI
OTHERS: M. Berman, Ph.D.	Chief, Laboratory of Mathematical Biology, DCBD, NCI	
D. Covell, Ph.D.	Staff Fellow	LTB NCI
COOPERATING UNITS (if any) S. Dower, IB, NCI A. Keenan, NM, CC R. Parker, S. Sieber, DCCP		
LAB/BRANCH Laboratory of Mathematical Biology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>We are injecting <u>monoclonal antibodies</u> reactive with normal cell types to investigate the <u>pharmacokinetics</u> of their distribution to target and non-target sites. Most of our studies to date have been done with a mouse IgG<sub>2a</sub> anti-H2 K<sup>k</sup>. It has been labelled with fluorescein for microscopy and fluorescence cell-sorting or labelled with <sup>125</sup>I for <u>gamma camera</u> imaging and organ isolation. Ferritin-labelled antibody will be used for electron microscopic correlation. We are also using a rat IgG anti-LY2 and several mouse IgG and IgM anti-Thy 1.1 and 1.2 antibodies. The initial aim is to delineate pharmacokinetic principles in well-defined, reproducible animal systems. Concurrently, we are establishing tumor models in mouse and guinea pig to test the possibilities for targeting to those abnormal cell types. SAAM models are being developed interactively with the experimental studies. This project is a recent outgrowth of work on the target-direction of liposomes by monoclonal antibodies.</p>		

Project Description:

Objectives: To investigate the pharmacokinetics of monoclonal antibodies in vivo for diagnosis and therapy of tumors.

Non-standard methods employed: (a) Fluorescence microscopy and fluorescence cell-sorting on monoclonal antibodies in vivo; (b) Image-correction techniques for quantitative analysis of gamma camera studies; (c) Use of SAAM modeling system to analyze the pharmacokinetics of antibody distribution and binding in vivo.

Significant findings: 1) Antibodies can be visualized selectively on very small aggregates of cells in the face of significant antigen expression on all cells of the animal; 2) Target cells become surface-labelled with fluorescent antibody.

Significance to Biomedical Research and the Program of the Institute: Radiolabelled monoclonal antibodies may be of major use in the diagnosis and staging of tumors. Monoclonals also may prove of therapeutic use in any of several ways: (i) by mobilizing body defenses against target cells; (ii) by delivering drugs, toxins, or radionuclides selectively to tumors; (iii) by "targeting" carriers (e.g., liposomes) for amplified effect of drug, toxin, or radionuclide. All of these endeavors require more pharmacokinetic information than is now available. We plan to develop that background in normal and tumor systems, with emphasis on the detection and treatment of metastases.

Proposed Course: Continue work with the murine histocompatibility antigens and T-cell markers; establish guinea pig hepatoma lines 1 and 10 as solid tumor models; use congenic retrovirus-associated murine B-cell lymphomas (expressing GP70) as a hematogenous model. In each of these systems we will combine fluorescence techniques (microscopy and cell sorting), radioisotope techniques (gamma camera and organ isolation), and ultrastructural techniques (ferritin-labelling) to investigate the anatomical, histological, and cytological localization of the antibodies. Against this background we will consider conjugation of toxins and/or liposomes to the antibodies and graduation to a clinically usable imaging nuclide, probably <sup>111</sup>In.

Publications:

Parker, R.J., Sieber, S.M., and Weinstein, J.N.: The effect of liposome encapsulation of a fluorescent dye on its uptake by the lymphatics of the rat. Pharmacology. 23: 128-136, 1981.

Weinstein, J.N., Leserman, L.D., Henkart, P.A., and Blumenthal, R.: Targeting of liposomes. In Gregoriadis, G., and Papahadjopoulos, D., (Eds.): Targeting of Drugs. N.Y., Plenum, in press.

Chused, T.M., Sharrow, S.O., Weinstein, J.N., Ferguson, W.J., and Sternfeld, M.: XRITC: A new dye for two-color immunofluorescence. J. Histochem. and Cytology. in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 CB 08360-01 LTB
PERIOD COVERED October 1, 1981 to September 30, 1982		
TITLE OF PROJECT (80 characters or less) Calculation of the Three-Dimensional Structures of Membrane-Proteins		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Matthew R. Pincus, M.D., Ph.D.      Expert, Theoretical Chemist, LMB NCI		
COOPERATING UNITS (if any)  R.D. Klausner, M.D. LBM, NIAMDD		
LAB/BRANCH Laboratory of Mathematical Biology		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NCI, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 0.25	PROFESSIONAL: 0.25	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>It is known that the primary (linear amino acid) sequence of a protein determines its <u>three-dimensional (tertiary) structure</u>. Potential functions have been developed that can be used to compute reliably the energies of any conformation of a given molecule especially in cases where solvation effects are negligible, as is the case for membrane proteins which require a low dielectric medium to insure proper <u>folding</u>. We have developed a method using these potential functions that allows for the computation of the three-dimensional structures of such proteins from their amino acid sequences and have applied it to the structures of leader sequences that cause transport of proteins across membranes and to the structure of the membrane- active protein, melittin.</p>		

Project Description:

Objectives: To calculate the three-dimensional structures of membrane proteins from their linear amino acid sequences and to relate their structures to their functions.

Methods Employed: We have developed a method called the method of combination of non-degenerate minima that allows us to build-up the conformation of a long polypeptide in segments. The method hinges on the identification of probable nucleation sites in the sequence. For membrane proteins, these sites are the long stretches of hydrophobic residues that occur in all of these proteins. Once these sites are identified, they are broken down into smaller di- and tripeptide segments, for which all of the allowed conformations are computed. The low-lying minima that have unique backbone (i.e., non-degenerate) conformations are selected for each of these segments and are combined with the corresponding allowed conformations for adjacent segments in the sequence. All of these resulting conformations are subjected to energy minimization, and the above procedure is repeated until the entire sequence is covered.

Major Findings: We have calculated the structures now of two membrane proteins: murine pre-kappa light chain leader sequence and melittin. In the first case, the sequence is Glu-Thr-Asp-Thr-(Leu<sub>3</sub>-Trp-Val)<sub>2</sub>-Pro-Gly. We have found that the first 8 hydrophobic amino acid residues exist in an alpha-helix that ends with a chain reversal at the terminal Trp-Val-Pro-Gly. The first 4 polar amino acids exist in a semi-extended conformation in which they are free to interact with a polar solvent. There is strong CD and ORD evidence that the hydrophobic core of leader sequences is alpha-helical and that ~50% of the molecule is in the alpha-form as we have calculated. Further, biological experiments in which leucine-containing leader sequences have beta-hydroxy-leucine (a strong helix-breaker) substituted for leucine indicate that these modified sequences are not functional.

We have also calculated the structure of melittin, the active component of bee-venom which intercalates into cell membranes to cause cell-lysis. The sequence is Gly-Ile-Gly-Ala-Val-Leu-Lys-Val-Leu-Thr-Gly-Leu-Pro-Ala-Leu-Ile-Ser-Trp-Ile-Lys-Arg-Lys-Arg-Gln-GlnNH<sub>2</sub>. We have found two alpha-helical structural domains (Val 5-Thr 10 and Pro 14-Ile 20) separated by a bend conformation involving the sequence Thr-Gly-Leu. Our calculated structure has an overall conformation of a bent helical rod with polar groups pointing outwards and non-polar side chains packing inward toward the interior of the molecule. These features are precisely the ones observed in the x-ray crystal structure of this molecule. Thus agreement between theory and experiment is excellent.

Significance to Biomedical Research and the Program of the Institute: We can now calculate the structures of simple membrane proteins from their amino acid sequences and can extend our methodology to compute structures for longer peptide chains. We are thus in a position to understand not only how these proteins function but also how mutations in their sequences lead to better structures or to loss of function.

Publications:

Pincus, M.R. and Klausner, R.D.: Prediction of the three-dimensional structure of murine pre-kappa light leader sequence. Biophys. J. 37: 95A, 1982.

Pincus, M.R. and Klausner, R.D.: Prediction of the three-dimensional structure of murine pre-kappa light leader sequence, a hexadecapeptide. Proc. Natl. Acad. Sci. U.S.A., 79: 3413-3417, 1982.

Pincus, M.R., Klausner, R.D., and Scheraga, H.A.: Calculation of the three-dimensional structure of the membrane-bound portion of melittin from its amino acid sequence. Proc. Natl. Acad. Sci. U.S.A., in Press, 1982.



















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